Lauric Arginate

Application for Listing in Schedule 1 of Standard 1.3.1 of the Australia New Zealand Food Standards Code

Substance:	Lauric Arginate
Applicant:	Laboratorios Miret, S.A. (LAMIRSA)
Ammendment Proposed:	Inclusion of Lauric Arginate in Schedule 1 of Standard 1.3.1 (Food Additives)
Assessment Procedure:	General

Prepared for

Laboratorios Miret, S.A. (LAMIRSA)

By

Competitive Advantage

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Executive Summary

Lauric arginate (ethyl-N^{α}-lauroyl-L-arginate HCl; CAS number 60372-77-2) is a new substance developed by Laboratorios Miret, S.A. for controlling microbial contaminants in a broad range of foods.

Lauric arginate has both good efficacy and low toxicity, making it a valuable tool in the prevention of food poisoning caused by contaminated foods.

Lauric arginate has demonstrated activity against bacteria, fungi and yeasts that contaminate foods. The efficacy of lauric arginate against contaminating microorganisms was confirmed in:

- Laboratory studies to determine the Minimum Inhibitory Concentration of the substance against a range of microorganisms, and
- Studies conducted with a variety of foods treated with lauric arginate and either naturally exposed to contaminating microorganisms or inoculated with specific microorganisms.

Studies in which lauric arginate was used in foods confirmed that lauric arginate is effective in preserving cheeses, meats, vegetables, beverages and other foods. While activity of lauric arginate in the vastly different matrices tested has demonstrated that the substance is effective in many different food types, it has also been determined that lauric arginate can react with casein in liquid milk to form a precipitate and thereby lose activity. Lauric arginate is not recommended for use in liquid milk although it can be used in a variety of milk products including cheeses (as demonstrated in the studies conducted).

In addition to being highly effective in controlling contaminating microorganisms, lauric arginate has low toxicity.

The low toxicity of lauric arginate was demonstrated through:

- Acute toxicity studies,
- *In vitro* and *in vivo* metabolism-toxicokinetics studies,
- *In vitro* mutagenicity assays,
- Subchronic toxicity studies,
- Chronic toxicity studies,
- Reproduction and developmental toxicity (one generation and two generations) and,
- Human kinetic studies.

The studies showed:

- Low acute oral toxicity (LD_{50} > 2000 mg/kg bw for both the parent compound and the primary metabolite [N^{α}-lauroyl-L-arginine or LAS]). In accordance with the definitions provided in the "Approved Criteria for Classifying Hazardous Substances" [NOHSC: 1008 (2004)], 3rd Edition, lauric arginate is not classified as being harmful if swallowed.
- Lauric arginate and its primary metabolite (N^{α}-lauroyl-L-arginine [LAS]) are not mutagenic

- No adverse effects in long-term studies, including reproduction studies, with the exception of some effects associated with the irritant nature of the concentrated solution.
- No significant abnormalities in any laboratory or clinical data obtained after adult male volunteers were give a single oral dose of 1.5 or 2.5 mg/kg bw ^{13}C-ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl as a 25% solution in propylene glycol.
- No lauric arginate was detected in adult male volunteers following administration of a single oral dose of 1.5 or 2.5 mg/kg bw ^{13}C-ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl as a 25% solution in propylene glycol. Metabolites declined to non-detectable levels within 12 hours demonstrating that ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl is hydrolysed into endogenous compounds.

Toxicokinetic and metabolism studies showed that after ingestion, lauric arginate is:

- Well absorbed,
- Rapidly metabolised,
- Is incorporated into naturally occurring products via the urea and citric acid cycle, and
- Is distributed to the liver and slowly excreted as carbon dioxide *via* expired air and, to a very low extent, *via* urine and faeces.

The safety of lauric arginate is further increased by its rapid degradation *in vivo* without generating breakdown/metabolic products likely to have adverse effects on health.

Based on the studies conducted, a NOAEL of 907 mg/kg bw/day (corresponding to 18,000 ppm, the highest dose administered) was adopted by the USA FDA. The same NOAEL is proposed for Australia.

JECFA reviewed lauric arginate at its 69th meeting held 17-26 June 2008 and used a NOAEL of 442 mg/kg bw per day from two reproductive toxicity studies to establish an ADI of 0-4 mg/kg bw.

With demonstrated activity against a broad range of microorganisms and low toxicity, Lauric arginate is considered to be an additional weapon in the arsenal available for fighting food poisoning, a problem estimated to cost Australia $1249 million per year. Its flexibility reduces the need for multiple food preservatives that are otherwise required in situations such as where variable pHs occur.

Lauric arginate does not have some of limitations associated with other common food preservatives e.g. unlike sorbic acid and benzoic acid, lauric arginate does not cause hypersensitivity in certain individuals. As a result, lauric arginate can be used as a replacement for other preservatives or it can be used in combination with other preservatives to provide required control of microbial contaminants without the adverse effects that may be associated with those other preservatives.

Lauric arginate is stable in both storage and in treated matrices further increasing its utility. The product has been shown to be stable at ambient storage conditions for up to two years.

In foods, lauric arginate can be hydrolysed, especially at high temperatures and low pH. However, as the conditions that favour most rapid hydrolysis are rare (pH < 3 with temperatures > 50°C for extended periods) are unlikely to occur in practice, it has been concluded that stability is not an issue in use of lauric arginate as a preservative in foods.

The benefits of lauric arginate have resulted in considerable interest from a number of potential users. These potential users have indicated interest in a variety of proposed uses

but, as with any new preservatives, they are unable to provide unqualified support for the product until they have thoroughly evaluated the product in their specific situations.

Foods in which lauric arginate could be used and the maximum proposed rates of use are summarised in the following table:

Item	***Food types***	***Lauric arginate (ppm; maximum)***
0.1	Preparations of food additives	225
1.6	Cheese - soft/cream/processed and mozzarella	450
1.6	Cheese – Hard/Semi-hard	1 mg/cm^2 of surface area of cheese.
1.4.2	Cream products (flavoured, whipped, thickened, etc)	225
2.2.2	Oil emulsions (<80% oil)	450
3	Ice confection sold in liquid form excluding milk based ice confections	225
4.1.3	Peeled and/or cut fruits and vegetables	225
4.3.1	Dehydrated legumes	225
4.3.4	Low joule jams	225
5	Confectionery products (e.g. chewing gums) but excluding chocolate and cocoa products	5625
6.4	Flour products (including noodles and pasta)	225
7	Breads and bakery products (surface treatment only)	450
9.3	Fish products	450
10.2	Liquid egg products (yolk preparations only)	450
11.4.1	Tabletop sweeteners – liquid preparation	115
12.6	Vegetable protein products	225
13.1	Infant formula products (in powder form)	2250
13.4	Formulated supplementary sports drinks	55
14.1.2	Fruit and vegetable juices and fruit and vegetable juice products (NOT apple juice)	55
14.3	Alcoholic beverages not included in item 14.2	55
14.1.3	Water based flavoured drinks and high energy drinks and soft drinks	55
14.1.5	Tea, herbal infusions and similar products (ready-to-drink only)	55
8.2, 8.3	Meat and meat products, including poultry	450
20.1	Beverages	55
20.2	Savoury toppings or fillings - essentially sauces such as tomato paste used in ready to eat pizzas, etc.	225 (vegetables) 450 (cheese)

Item	***Food types***	***Lauric arginate (ppm; maximum)***
20.2	Dairy and fat based desserts, dips and snacks Soup bases (made up as directed)	450

Approvals for use of lauric arginate in foods are being sought globally. A comprehensive data package, including an analytical method using reverse-phase, high-performance liquid chromatography developed to quantify the content of lauric arginate present in different food matricies, has been prepared.

Submission of the data package has enabled lauric arginate to be approved for similar uses to those proposed in Australia in the USA and in Mexico. The data have been submitted to the European Union for authorisation of lauric arginate as a new additive for use in food for human consumption. Approval of the application in the EU is still pending.

Available data were assessed by JECFA which established an ADI of 0-4 mg/kg bw for lauric arginate used in food.

The US FDA has agreed to classification of lauric arginate as Generally Recognized As Safe (GRAS). Lauric arginate is also approved for use in Mexico.

Lauric Arginate

Application for Listing in Schedule 1 of Standard 1.3.1 of the Australia New Zealand Food Standards Code

General Information on the Application

Applicant Details

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Nature of Applicant's Business

The company has two main business areas:

- Manufacture of food additives.
- Manufacture of chemical products to be applied as biocides in woods, leather, paints, paper and other industrial fields.

Details of other individuals, companies or organisations associated with the application

Laboratorios Miret, S.A. is part of Grupo Lamirsa. Venta de Especialidades Químicas S.A. (VEDEQSA) is also a member of Grupo Lamirsa.

As the parent company of Laboratorios Miret, S.A., Grupo Lamirsa is associated with the application as are the other Grupo Lamirsa subsidiary (Venta de Especialidades Químicas S.A.).

There are no other individuals, companies or organisations with any rights to this application.

The application is prepared and submitted to FSANZ on behalf of Laboratorios Miret, S.A. (LAMIRSA) by:

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Purpose of the Application

The purpose of this application is to obtain listing of a new food additive (ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl which is also known as lauric arginate or LAE) in Schedule 1 of Standard 1.3.1 (Food Additives) of the Australia New Zealand Food Standards Code.

Lauric arginate is a new patented preservative (Appendix 68) of natural origin with good efficacy against a broad range of microorganisms and low mammalian toxicity.

Justification for the Application

Technological Function of the Food Additive

Lauric arginate is an innovative food preservative for use in a wide variety of foods including drinks, meats, fish products, pastas, rice, fruit and vegetable products (e.g. ready-to-eat salads) and savoury toppings and fillings.

Lauric arginate can be used alone or in combination with other preservatives.

Alone, lauric arginate can replace benzoates, especially in food matrices with high pH.

Lauric arginate can also be used with preservatives such as sulphites to reduce the level of these other preservatives where otherwise, the levels of preservatives such as sulphites may be unacceptably high.

Lauric arginate has a broad range of antimicrobial properties. The spectrum of activity is illustrated in the table of Minimum Inhibitory Concentrations for lauric arginate:

Table 1: Minimum Inhibitory Concentration of Lauric Arginate against Different Microorganisms

Gram Positive Bacteria	Lauric Arginate (µg/mL)
Arthrobacter oxydans ATCC 8010	64
Bacillus cereus var mycoide ATCC 11778	32
Bacillus subtilis ATCC 6633	16
Clostridium botulinum ATCC 19397	64
Clostridium perfringens ATCC 77454	16
Clostridium perfringens ATCC 12917	16
Lactobacillus curvatus ATCC 25601	16
Lactobacillus delbrukeii sp lactic ATCC 10705	16
Lactobacillus paracasei ATCC 25302	16
Lactobacillus plantarum ATCC 8014	16
Listeria monocytogenes B4/97	8
Leuconostoc mesenteroides ATCC 19255	32
Micrococcus luteus ATCC 9631	128
Micrococcus luteus ATCC 9631	2
Staphylococcus aureus ATCC 6538	8

Gram-Negative Bacteria	Lauric Arginate (µg/mL)
Alcaligenes faecalis ATCC 8750	64
Bordetella bronchiseptica ATCC 4617	128
Campylobacter jejuni ATCC 29428	8
Campylobacter jejuni HC	16
Citrobacter freundii ATCC 22636	64
Enterobacter aerogenes ATCC 13048	32
Enterobacter aerogenes ATCC 13048	4
Enterobacter sakazakii ATCC 29544	32

Gram-Negative Bacteria	Lauric Arginate (µg/mL)
Escherichia coli ATCC 8739	32
Klebsiella pneumoniae var. pneumoniae ATCC 4352	32
Proteus mirabillis CECT 170	32
Pseudomonas aeruginosa ATCC 9027	32
Pseudomonas fluorescens ATCC 13430	32
Salmonella choleraesuis ATCC 13076	8
Salmonella typhimurium ATCC 14028	32
Shigella dysentariae ATCC 13313	8
Serratia marcescens ATCC 10759	32
Yersinia enterocolitica ATCC 27729	16
Vibrio parahaemoliticus ATCC 17802	128

Studies conducted at the Department of Microbiology, University of Barcelona (full details provided in Confidential Appendix 1)

In addition to activity against bacteria, lauric arginate has been shown to be active against important fungi and yeasts. Full details are provided in Confidential Appendix 1-3.

The mode of action of lauric arginate is to alter the metabolic processes of susceptible micro-organisms by inducing changes in the membrane potential of target cells without causing cell lysis (Appendix 1).

While understanding of the mode of action of an antimicrobial substance and its Minimum Inhibitory Concentration (MIC) provides useful indications of activity, it is also necessary to demonstrate that the required level of activity is achieved when the product is used in target food matrices.

An example of potential problems was given in early research with lauric arginate which showed the substance was capable of reacting with certain elements in selected matrices, e.g. in milk, lauric arginate reacts with casein to form a precipitate which does do have the antimicrobial properties of lauric arginate. Consequently, lauric arginate is not recommended for use in milk.

Studies were subsequently conducted to evaluate the efficacy of lauric arginate in different foods.

Efficacy of Lauric Arginate in Cheese

The early results that indicated lauric arginate could be inactivated in liquid milk suggested a need for evaluation of the product in other dairy products, e.g. cheeses. These studies showed that lauric arginate is very effective in preventing contamination of cheeses.

With hard and semi-hard cheeses (e.g. parmesan) moulds and contaminating microorganisms develop on the outside of the block of cheese.

Effective control of surface moulds, etc can be achieved if an antimicrobial barrier is placed over the cheese.

To test the penetration, uptake or dispersion of lauric arginate into a block of hard/semihard cheese, a study was conducted in which different cheeses were dipped in a 1% or a 5% solution of lauric arginate. 1 mm thick slices were then taken from the outside of the treated blocks moving towards the centre. In most cases, lauric arginate was found only in the first 1 mm slice. In the case of Grana Padano cheese, the crumbly

nature of the cheese prevented 1 mm thick slices being taken and it was necessary to take 3 mm thick slices.

Lauric arginate was normally found to migrate into the cheese to a short distance only. It was generally found in the first slice, i.e. in most cheeses in the first 1mm, indicating the product remained as a barrier against infection for surface contaminating microorganisms.

Full details of the study are provided in Confidential Appendix 4.

Softer cheeses can be more easily contaminated and are therefore considered to be more difficult media for testing antimicrobial additives than the harder cheeses. At the same time, the higher moisture content of such cheeses could be considered to make lauric arginate susceptible to interaction and inactivation within the cheese. To test the activity of lauric arginate in soft cheeses, studies were conducted using ricotta, mozzarella and mascarpone cheese.

In the case of mascarpone cheese, lauric arginate was added during production of the cheese. Lauric arginate was added at 200 ppm and 500 ppm in a formulated product.

Full details of the study are contained in Confidential Appendix 5.

The study showed:

- Lauric arginate reduced the total count of aerobic mesophiles for at least 35 days compared with an untreated control.
- Increasing the concentration of lauric arginate resulted in greater suppression of aerobic mesophile counts.
- Organoleptic tests conducted using trained analysts showed no adverse effects on the characteristics of the cheese.

In the case of mozzarella cheese, lauric arginate was added to the "liquido di governo" (i.e. the water-based liquid in which mozzarella cheese is normally presented) at a concentration of 200 ppm. Full details of the study are provided in Confidential Appendix 6.

Some of the mozzarella cheese was inoculated with *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Escherichia coli* while other samples were not inoculated.

Like in the mascarpone cheese, there was a marked reduction in aerobic mesophiles in the non-inoculated samples. In the inoculated samples, there was significant reduction in *Enterobacter* and *Pseudomonas* counts but the low incidence of *E. coli* in the untreated controls made conclusions about efficacy against *E.coli* not possible.

No adverse effects were observed in testing of organoleptic characteristics by trained analysts.

A further study was conducted in which ricotta cheese was inoculated with *Listeria monocytogenes* (Confidential Appendix 7). Lauric arginate produced a significant reduction in *L. monocytogenes* numbers for more than 8 days.

The cheese studies showed that lauric arginate is effective in controlling a range of contaminating bacteria in cheeses, even though those cheeses may contain substances that in liquid milk are known to reduce the efficacy of lauric arginate.

A further study (Confidential Appendix 8) in which lauric arginate was applied onto gorgonzola cheese as well as onto equipment used in handling the cheese. Lauric arginate

was applied onto fresh cheese, mature cheese or both fresh and mature cheese. The results showed:

- Lauric arginate reduces mesophilic aerobes and *Listeria* spp.
- Best results are obtained when lauric arginate is applied onto fresh and mature cheese.

The gorgonzola study (Confidential Appendix 8) confirmed the efficacy of lauric arginate but also highlighted the need to keep microbial populations under control by reducing/suppressing populations on the cheese throughout the product process.

Efficacy of Lauric Arginate in Vegetables

A totally different substrate/matrix is vegetables.

Studies have been conducted in various vegetables to assess the effectiveness of formulated products containing lauric arginate.

Studies were conducted in vegetables (carrots and chickpeas) as well as in prepared salads.

Chickpeas are a common vegetable in certain diets and are an important ingredient in many ready-to-eat meals.

Normally, chickpeas need to be soaked in water for 12-24 hours at room temperature before cooking. The soaking process offers an opportunity for contamination, including initiation of fermentation.

A study (Confidential Appendix 9) was done to evaluate the ability of lauric arginate to inhibit fermentation and prevent microbiological contamination. Lauric arginate (100 mg/kg) was compared with an untreated control.

The increase in total viable counts in the chickpeas treated with lauric arginate was markedly less than in the untreated control (0.3 $\log_{10}$ CFU/g versus 1.3 $\log_{10}$ CFU/g respectively). Similar inhibition was observed in the soaking bath waters.

A further study was conducted in which lauric arginate was added to a bath in which carrots were immersed. Counts of aerobic mesophiles were done for 9 days after treatment.

The results showed a significant reduction in mesophilic aerobes for at least 9 days after treatment (Confidential Appendix 10).

Cutting vegetables to prepare salads increases the probability of contamination as a result of the salad preparation process. As a result, a study was conducted in which salads were prepared according to normal European commercial salad preparation practices. Lauric arginate was compared against an untreated control and a sodium benzoate and potassium sorbate treatment. Lauric arginate was used at 200 ppm. Full details of the study are provided in Confidential Appendix 11.

The results of the study showed:

- Lauric arginate provided similar suppression of aerobic microorganisms and enteric bacteria at a similar level to that of the sodium benzoate plus potassium sorbate treatment.
- Lauric arginate provided significant reductions in aerobic microorganisms and enteric bacteria counts compared with the untreated control, especially from 22 days after study initiation.

- No adverse effects on organoleptic properties were observed.

Efficacy of Lauric Arginate in Meat

Meat is another matrix that can support contaminating microorganisms while at the same time adversely affecting performance of antimicrobial substances.

A study was conducted in which lauric arginate was introduced into shrink-wrap bags immediately prior to introducing ham and sealing of the bags. Prior to placement in the bags, the hams were surfaced inoculated with *L. monocytogenes*. Lauric arginate was applied as either a 5% or 10% solution in distilled water.

Full details of the study are provided in Appendix 12.

The study showed that:

- Increasing the concentration of lauric arginate produced a higher reduction in CFU/ham.
- With a 5% solution of lauric arginate, adding a higher volume of diluted product produced greater reductions in CFU/ham.
- The pathogen population was reduced to below the limit of detection within 24 hours of storage at 4°C when a 5% solution of lauric arginate was placed in a bag in which the ham had been inoculated with 3.0 log CFU/ham.
- Pathogen levels remained below the limit of detection for more than 60 days in samples treated with 8 mL of a 5% lauric arginate solution but numbers increased by about 2.0 log CFU/ham when volumes of the 5% solution were less than 8 mL per bag.

The study provided in Appendix 12 shows lauric arginate to be effective against a major microbial contaminant of meat in shrink-wrap.

Ready-to-eat meals are becoming more popular and meat is frequently a component of the meal, including as a component in toppings and fillings used in such prepared meals.

Stewed veal was prepared, cooled and vacuum sealed in sterile plastic bags. 100 mg/kg lauric arginate treatment was compared against an untreated control. The stewed veal was inoculated with a mixture of bacteria and yeasts including *Escherichia coli*, *Enterobacter aerogenes*, *Staphyloccocus aureus*, *Bacillus cereus*, *Candida albicans* and *Saccharomyces bailii*.

The results confirmed lauric arginate suppresses bacterial population development relative to that of the untreated control (Confidential Appendix 12). The shelflife for the stewed veal was increased from less than 14 days to approximately 1 month by treatment with lauric arginate.

Another study investigated the activity of lauric arginate in marinated meat (Confidential Appendix 13).

In this study, lauric arginate (180 mg/kg) was added by dipping fresh beef into a bath containing lauric arginate. Lauric Arginate was compared against a sodium nitrate treatment and an untreated control.

The results showed that lauric arginate suppressed mesophilic aerobes greater degree than the sodium nitrate treatment. Both applied treatments suppressed aerobic mesophiles relative to the untreated control. No adverse effects on organoleptic characteristics were observed. It was concluded that lauric arginate was a suitable replacement for nitrates in controlling contaminating microorganisms in marinated meat.

Lauric arginate has also been shown to be effective in controlling contaminating microorganisms in poultry meat. A study in which smoked turkey slices were topically treated by immersion in a lauric arginate solution (Confidential Appendix 14) and inoculated with *Listeria monocytogenes* showed lauric arginate markedly suppressed development of the *L/ monocytogenes* population compared with the untreated control. Similarly, total Aerobic Plate Counts (APC) were markedly lower in the lauric arginate treated meat relative to the untreated control and remained low during 8 weeks of storage at 4.4°C. The study director concluded that lauric arginate has the potential to extend the microbial shelflife of refrigerated sliced smoked turkey. Similar results were obtained with roast turkey slices (Confidential Appendix 15).

Cured ham slices were also treated in a similar fashion to the poultry slices. As with the poultry slices, marked reductions in *L. monocytogenes* numbers were observed in the lauric arginate treated samples compared with the untreated controls. Lauric arginate also inhibited the growth of aerobic plate counts developing on the samples during storage at 4.4°C. The study director concluded that lauric arginate has the potential to extend the microbial shelflife of refrigerated sliced cured ham.

Another type of meat product is sausages.

Bratwurst sausages were treated with 100 mg/kg lauric arginate during manufacture of the sausages.

Compared with the untreated control, lauric arginate significantly reduced total aerobic bacteria on the surface of the sausages for up to 90 days. There was no difference in the microbial count inside lauric arginate treated sausages compared with untreated sausages.

Lauric arginate significantly reduced numbers of' *Clostridium* sp in and on the sausages relative to the untreated controls.

Efficacy of Lauric Arginate in Fish

Salted fish (e.g. salted cod) needs to be desalted before consumption. Removal of the salt is achieved by soaking in water for two to three days.

To evaluate the effectiveness of lauric arginate in preventing microbial contamination during the salt removal phase, lauric arginate was added to the de-salting bath of either 80 mg/kg or 160 mg/kg. These treatments were compared against an untreated control. Full results are provided in Confidential Appendix 16.

Within 9 days after de-salting the untreated controls were observed to have a bad odour and an unacceptable colour. In contrast, lauric arginate treated samples retained their characteristic colour and odour. In addition, total viable counts were significantly lower in the lauric arginate treated samples than in the untreated controls.

It was concluded that lauric arginate was effective in retarding microbiological spoilage of dried cod without affecting the organoleptic properties of dried cod.

Another study was conducted to evaluate the ability of lauric arginate preserved fish roe products without affecting their technological characteristics. Full details of the study are provided in Confidential Appendix 17.

In the fish roe study, lauric arginate was added to the fish roe at 200 mg/kg. Lauric arginate was compared with benzoic acid and an untreated control.

Staphylococcus aureus, *E. coli* and total viable counts were significantly reduced by both lauric arginate and benzoic acid treated roe compared with the untreated control for at

least nine days (duration of the study). For both the applied preservatives, numbers of contaminating microorganisms declined over the course of the study (nine days) whereas they increased in the untreated control.

The above discussed studies confirm that lauric arginate is effective in controlling contaminating microorganisms in and on a range of meat and fish products. The end result is that the normal shelflife of such meat products can be extended in many situations.

Efficacy of Lauric Arginate in Processed and Other Foods

In addition to the extremes of the meats, cheeses and vegetables, lauric arginate has been evaluated in a broad range of prepared foods:

- In **pastries and bakery products**, moulds tend to develop on the outer surface. A study in which doughnuts were surface treated with lauric arginate showed significant reduction in mild growth compared with the untreated control (Confidential Appendix 18). A further study in which a cream pastry was treated with 1% or 3% lauric arginate showed good efficacy relative to the to control (Confidential Appendix 19). Development of microorganisms was suppressed for at least 40 days.
- A study was conducted to evaluate the ability of lauric arginate to preserve **cooked pasta** when used in ready-to-eat meals and prepared salads (Confidential Appendix 20). Lauric arginate (100 mg/kg) was compared against potassium sorbate and an untreated control. The prepared pasta was then stored under refrigeration (4°C). Lauric arginate significantly reduced total viable counts relative to the untreated control. It was estimated that total shelf life could be extended by more than five days when pasta was treated with 100 ppm lauric arginate.
- As with pasta, **cooked rice** is used in ready-to-it meals and prepared salads. A study was conducted to evaluate the ability of lauric arginate to preserve cooked rice for use in ready-to-eat meals and prepared salads (Confidential Appendix 21). Lauric arginate was used at 100 mg/kg and 300 mg/kg. It was compared with potassium sorbate and an untreated control. The cooked rice was stored under refrigeration (4°C) and at 20°C. By day 4 at 20°C, both the sorbate treated samples and the untreated control were observed to have a bad odour and an unacceptable colour. Similarly, within 11 days at 4°C, the untreated control samples were observed to have a bad odour and an unacceptable colour. In contrast, the lauric arginate treated samples maintained their characteristic odour and colour in both cases. Total viable counts were significantly reduced in the lauric arginate treated samples compared with both the potassium sorbate and untreated samples.
- The ability of lauric arginate to suppress microbial contamination in **refrigerated soups** was evaluated (Confidential Appendix 22). Lauric arginate was added at 0.2 g/kg and compared with an untreated control. Lauric arginate significantly reduced the count of aerobic mesophiles for up to 30 days (duration of study). Evaluation of organoleptic properties by trained analysts showed no effect of the addition of lauric arginate on organoleptic properties.
- The ability of lauric arginate, when added at rates of 0.1 - 1.1 g/kg, to suppress microbial contamination in **sauces** was evaluated in guacamole (Confidential Appendix 23). There were no adverse effects on organoleptic properties of guacamole treated with lauric arginate at rates of 0.3 g/kg or less. While 0.1 g/kg lauric arginate did not significantly suppress aerobic mesophiles compared with the untreated control, all other rates did suppress aerobic mesophiles compared with the untreated control. Aerobic mesophile populations continued to decline through the 12 days of the study in samples to which lauric arginate at greater than 0.1 g/kg had been added. Similarly, *E. coli*, *Salmonella typhymorium* and *Listeria moncytogenes* populations continued to decline throughout the course of the study (12 days).
- A study was conducted to evaluate the effectiveness of lauric arginate in preserving **fresh tomato sauce** (Confidential Appendix 24). Samples were treated with 200

mg/kg lauric arginate and compared with samples treated with sorbic acid and an untreated control. Samples were stored under refrigeration (0-5°C) for 25 days. Both sorbic acid and lauric arginate suppressed total viable counts and yeasts significantly compared with the untreated control. No adverse effects were observed on organoleptic properties from addition of lauric arginate to the tomato sauce.

- Lauric arginate was tested in **pizza toppings** to determine its efficacy in controlling spoilage organisms (Confidential Appendix 25). Lauric arginate was applied at 200 mg/kg and was compared against a potassium sorbate treatment and an untreated control. Both potassium sorbate and lauric arginate significantly suppressed total viable counts over the 14 day study period. Lauric arginate suppressed total viable counts to a greater degree than potassium sorbate.
- The effect is of lauric arginate in suppressing microbial contaminants in **non-alcoholic flavoured drinks** was tested in a study using carbonated orange juice drink (Confidential Appendix 26). Lauric arginate at 100 mg/kg was compared with benzoic acid treatment and with an untreated control. Samples were inoculated with either *Candida albicans* orl *Lactobacillus plantarum*. Samples were stored at 22°C. Lauric arginate significantly reduced *C. albicans* and *L. plantarum* whereas benzoic acid had minimal effect on both these populations. It was concluded that lauric arginate is a superior preservative in orange juice drinks than benzoates.
- The ability of lauric arginate to control microbial contaminants in a **non-carbonated fruit drink** was evaluated in a study in which lauric arginate at 40 mg/L, 50 mg/L or 100 mg/L was added to a noncarbonated cranberry/raspberry juice drink and a noncarbonated kiwi/strawberry juice drink (Confidential Appendix 27). Lauric arginate was compared against an untreated control. The drinks were inoculated with either *Candida albicans* or *Saccharomyces bailii*. All applied treatment significantly reduced the target microorganisms relative to the untreated control for the duration of the study (3 days).
- The effect of lauric arginate on microbial contaminants in **fruit based concentrates** was evaluated in a study in which lauric arginate was added to grape concentrate at 100 mg/kg (Confidential Appendix 28). Lauric arginate was compared with an untreated control. The results showed that lauric arginate significantly reduced yeasts compared with the untreated control. It was concluded that lauric arginate was an effective preservative for grape concentrates.
- Lauric arginate was evaluated as a preservative in **sports drinks** (Confidential Appendix 29). Lauric arginate at 50 and 100 mg/L was compared with a benzoic acid plus sorbic acid treatment and an untreated control. The drinks were inoculated with *Candida albicans*, *E. coli* and *Aspergillus niger*. All applied treatments significantly reduced *Candida albicans*, *E. coli* and *Aspergillus niger* numbers relative to the untreated control for the duration of the study (9 days). 50 mg/L lauric arginate was not as effective as 100 mg/L lauric arginate in suppressing *Aspergillus niger* populations.

The studies discussed above show lauric arginate is effective against a broad range of contaminating microorganisms and is suitable for use in many food matrices, including:

- cheeses,
- meats,
- fish,
- vegetable materials,
- salads and salad ingredients, and
- beverages

The matrices used in the studies discussed above provide a broad range of situations to test the limits of the product. These tests have shown that lauric arginate would be effective in most food matrices.

Safety of the Food Additive

Extensive testing of lauric arginate, a food preservative with excellent inhibition of microorganisms, derived from naturally based compounds, has shown the product to be of low toxicity.

Lauric arginate is highly effective in controlling many pathogenic microorganisms that cause serious illnesses in humans including *Listeria monocytogenes*, *Salmonella sp*. and *Escherichia coli* as well as other bacteria, algae and fungi (Confidential Appendices 1-3). Lauric arginate is typically formulated as solutions dissolved in appropriate food-grade solvents (e.g. propylene glycol, glycerol) containing 20-25% lauric arginate (although the percentage of lauric arginate could vary according to needs).

The ability of lauric arginate to provide protection from contaminating microorganisms in prepared foods combined with its low toxicity indicates lauric arginate can be used as a component in food safety programmes.

The low toxicity of lauric arginate was demonstrated through:

- Acute toxicity studies,
- *In vitro* and *in vivo* metabolism-toxicokinetics studies,
- *In vitro* mutagenicity assays,
- Subchronic toxicity studies,
- Chronic toxicity studies,
- Reproduction and developmental toxicity (one generation and two generations) and,
- Human kinetic studies.

Studies were conducted in accordance with OECD guidelines for the testing of chemicals and the relevant recommendations as set out in Directives of the European Commission and in accordance with the principles of Good Laboratory Practice (GLP) described in EU Council Directive 87/18/EEC.

Toxicological studies were performed with:

- Lauric arginate with a purity of 85-95% ethyl-N^{α}-lauroyl-L-arginate HCl.
- Mirenat-N, the formulated product in which lauric arginate is dissolved in propylene glycol, and
- N^{α}-lauroyl-L-arginine (LAS) which is the first metabolite of lauric arginate.

Although most of the studies are considered to be confidential for commercial reasons, a number of papers based on the studies have been published in the following scientific journals:

1. Food and Chemical Toxicology, and
2. Journal Applied of Microbiology.

Acceptable Daily Intake (ADI)

NOAELs (No Observed Adverse Effect Levels) were derived from results obtained with subchronic, chronic, embryo-foetal and fertility studies.

Table 1 summarises the NOAEL values determined in various toxicological studies (expressed as mg/kg bw/day of lauric arginate):

Table 1: *Summary of NOAELs obtained in different toxicological experiments expressed as lauric arginate mg/kg bw/day*

Experiment	Test substance	NOAEL (mg/kg bw/day)	Animal tested
Subchronic toxicity	Lauric arginate	1143	Male rats
		1286	Female rats
Subchronic toxicity	Mirenat-N	718	Male rats
		848	Female rats
Chronic study	Lauric arginate	307	Male rats
		393	Female rats
One generation reproductive and toxicological studies	Lauric arginate	2000	Rats (Dams)
		2000	Rats (Foetuses)
		300	Rabbits (Dams)
		1000	Rabbits (Foetuses)
Two generation reproductive performance study	Lauric arginate	1073	Male and female rats

An initial review of the data provided in Table 1 suggests a NOAEL of 307 mg/kg bw/day for local gastric irritation in male rats in the chronic feeding study be used in determining the ADI. However, gastric irritation is not normally considered to be an "adverse effect".

"Adverse effect" is defined by JECFA as "Change in the morphology, physiology, growth, development, reproduction or life span of an organism, system, or (sub) population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences" (Glossary of Terms, Principles and Methods for the Risk Assessment of Chemicals in Food, Draft dated May 2008). Gastric irritation does not satisfy this definition and, therefore, it is concluded that gastric irritation should not be used as the basis for setting the NOAEL.

The USA FDA similarly concluded that gastric irritation should not be used as the basis for establishing the NOAEL. Consequently, in the USA, 907 mg/kg bw/day (corresponding to 18,000 ppm, the highest dose adminstered) was adopted as the NOAEL.

Mutagenicity studies showed that lauric arginate, Mirenat-N (formulated product containing lauric arginate) and LAS (degradation product) did not have mutagenic effects in bacterial systems, mammalian cells and did not show clastogenic activity in mammalian chromosome aberration tests (Appendices 2-9).

The only "toxicity" observed in the sub-chronic, chronic and reproductive toxicity tests was due to the irritant nature of the concentrated solutions (Appendices 10-15). The observed effects are not considered "adverse".

Metabolism
The metabolism of lauric arginate was compared between laboratory animals and humans to validate the toxicological results obtained and to help explain the very low toxicity of lauric arginate (Appendix 16).

The metabolism-toxicokinetic studies showed the active ingredient of lauric arginate, ethyl-N^{α}-lauroyl-L-arginate HCl, is rapidly metabolised to LAS, ethanol, arginine and lauric acid in both rodents and humans (Appendices 17-22). The results demonstrated that the exposure to lauric arginate *in vivo* was likely to be very short and independent of the solvent used for its formulation (Appendix 23).

Lauric arginate is rapidly degraded without generating break-down/metabolic products likely to have adverse effects on health.

Carcinogenicity
The:

- rapid metabolism of ethyl-N^{α}-lauroyl-L-arginate HCl to natural compounds found in the diet,
- absence of significant systemic toxic effects in the studies performed, and
- minor nature of the findings in the clinical human studies,
- together with the absence of mutagenic activity,

does not suggest any carcinogenic potential.

Consequently, it is not considered necessary to perform carcinogenicity studies.

Conclusion
In conclusion lauric arginate is of low toxicity, posing minimal/no risk to consumer health when used according to directions as food preservative.

Costs and Benefits for Industry, Consumers and Government

Abelson, *et. al* (2006) estimated that the total cost of food poisoning is $1,249 million per year in Australia (Appendix 24). This includes a cost of $231.5 million for premature mortality and $221.9 million for health care services. The authors further stated that the problem with food poisoning is likely to increase as the Australian population ages (and becomes more vulnerable).

The sizable cost to the community as a whole can be much more significant on individual companies, which may incur large costs for recalling contaminated goods (estimated at >$1 million for a product sold nationally through supermarkets) as well as for other companies within the industry. As an example, a Queensland Department of State Development and Department of Employment, Training and Industrial Relations review of the Queensland meat processing industry training needs (published in 2000; Appendix 25), in discussing the structure of the industry, noted that the small goods sector had, at the time of the review, just started to recover from the "Garibaldi mettwurst food poisoning incident" some five years previously. The cost to other companies of contamination and ultimate closure of Garibaldi is not known.

In addition to companies, individuals can suffer as a result of food contamination. Consumers eating contaminated food can become sick resulting in reduced productivity and enjoyment of recreational and family activities. Employees of a company producing goods found to be contaminated can suffer through loss of income (e.g. loss of overtime) as production is scaled back whilst the contamination is investigated and rectified. In the case where the company is forced out of business (as occurred with Garibaldi), employees can lose their livelihood. Some employees can even face prosecution for their involvement in food poisoning incidents. Loss of consumer confidence can adversely affect the income of retailers and producers (e.g. when seafood from certain areas is considered to be contaminated).

There are numerous methods available to help manage and minimise potential contamination. Food preservatives are one of the tools used for this purpose, as confirmed by FSANZ in its report of the 21st Australian Total Diet Study when it stated that "sulphite, benzoate and sorbate preservatives are widely used throughout the food industry".

While preservatives such as sulphites, benzoates and sorbates are available, they have limitations. Limitations of some of the common preservatives include:

1. Salts of sorbic acid and benzoic acid are only effective at low pH. Consequently, these salts cannot be used against microorganisms that can grow in neutral or basic pH environments.

2. Sorbic acid and benzoic acid have been found to be capable of inducing hypersensitivity in some individuals.

3. Sulphites have the potential to cause severe reactions in some individuals, particularly those suffering from asthma.

4. Nitrite compounds (e.g. sodium nitrite) have been shown to be capable of forming mutagenic, teratogenic and carcinogenic nitrosamines in the presence of amines and amides under certain conditions.

5. Ingestion of nitrites by infants can induce the formation of critical levels of methaemoglobin. In addition, pregnant women and people with metabolic disorders may be more sensitive and predisposed to nitrite-induced methaemoglobinaemia.

6. Nitrates, at pH greater than 7.5, promote rather than inhibit bacterial growth; at a pH between 6.0-7.0, the antibacterial effect is very much reduced;

7. Sensitivity to pH, e.g. nitrates at a pH between 4.5-5.5 have good anti-microbial activity but below 4.5, activity is reduced.

8. Activity against certain organisms only e.g. nitrates are not effective in controlling Gram negative pathogens in commercially prepared foods.

9. Interference from contaminants e.g. the protective effect of nitrates is reduced in products with high iron content such as those containing liver or blood.

10. Formation of toxic or otherwise unacceptable products e.g. nitrates added to meat can be gradually transformed into nitrites and then, a portion can be transformed into nitrosamines.

11. Potential adverse effects e.g. nitrates have been implicated in thyroid disease and elevated doses have been shown to induce methaemoglobin formation in infants.

12. In Australia, FSANZ has determined (Proposal P298: Benzoate and Sulphite Permissions in Food) that high-level consumers may exceed the ADI for benzoates and sulphites. In particular, children aged 2-5 years were particularly at risk.

Lauric arginate provides an alternative to these food preservatives since:

1. Lauric alginate is effective not only at acid pHs but also at near-neutral pH values.

2. The interaction between lauric arginate and other compounds present in foods (hydrocolloids, antioxidants, colour additives, proteins and protein extracts) did not result in the formation of N-nitroso compounds (Appendix 67).

3. Lauric arginate can replace the use of benzoates and can be used in combination with sulphites to reduce the amount of sulphites used in foods.

In addition, lauric arginate provides further benefits to industry, consumers and government. These benefits include:

1. A flexible yet effective food preservative that can be used over a broad range of pHs without inducing adverse effects associated with other commonly available food preservatives. This gives the food industry a more flexible tool than provided by the other commonly available preservatives.

2. Effective protection of foods from contaminating microorganisms under a broad range of conditions. Consumers benefit through reduced exposure to potentially harmful contaminating microorganisms.

3. Maintenance of confidence in food industries by minimising the potential for food poisoning outbreaks. This benefits the food industry as a whole as well as other industries that do not incur the cost of having employees off-work due to food poisoning episodes. Government benefits through the potential for consumer dissatisfaction with food safety programs being reduced and the food industry benefits through obtaining a more flexible food preservative that has minimal adverse effects when used in accordance with recommendations.

In summary, lauric arginate provides a flexible tool as part of the range of tools available to combat food poisoning, the cost which has been estimated to be $1,249 million per year. In addition, the flexibility of lauric arginate gives those companies using the product

potential benefits through reduced need to use multiple food preservatives due to factors such as pH variability as a result of the flexibility of lauric arginate. There are further potential benefits for small business in having a flexible food preservative that can be used in ready to serve meals such as fruit salads and take-away pasta dishes. Consumers benefit through the use of a low toxicity yet effective food preservatives within the overall contamination management environment employed in the manufacture of the foods they consume.

Support for the Application

Lauric arginate is a food preservative. Food preservatives are widely used in the food industry and are considered essential in many food types. Consequently, it can be concluded that there is widespread support for the use of food preservatives in general within the food industry.

The specific food additive lauric arginate has been discussed with only a limited number of potential users. Users have indicated an interest in the product, e.g. see comments from some prospective users in Confidential Appendix 30. However, until a product is commercially evaluated by individual companies, they are not able to provide unequivocal support for any ingredient, including lauric arginate.

Internationally, there is concern about many of the commonly used preservatives. As an example, Coca-Cola has announced that it will be phasing out sodium benzoate in some of its drinks due to "demand for more natural products" (see Appendix 26). The attached article (Appendix 26) mentions that Coca-Cola will be removing sodium benzoate from more products "where technically possible".

Lauric arginate makes it technically possible to replace sodium benzoate in many situations.

Many food companies undertake market research to understand the preferences of their customers. The article in Appendix 26 points out that while there a safety concerns about preservatives such as sodium benzoate, the decision to replace sodium benzoate in certain Coca-Cola brand products is driven by "consumer preferences for natural" ingredients. Lauric arginate is a naturally derived product that potential customers have expressed interest in but need to evaluate using their own in-house testing procedures before they can make a decision to switch from currently used preservatives to lauric arginate.

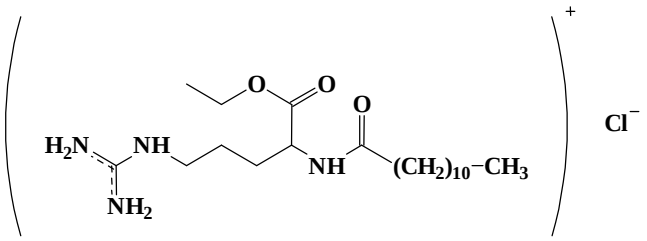
Technical Information on the Food Additive

Nature and Technological Function of the Additive

Identification of the Additive

The active ingredient of lauric arginate is the hydrochloride salt of an N-fatty acyl-substituted amino acid ethyl ester with the chemical name ethyl-N$^{\alpha}$-lauroyl–L-arginate HCl. Lauric arginate, which is manufactured by Laboratorios Miret S.A. (LAMIRSA), is a white powder proposed to be used for food preservation. The product contains between 85-95% of the active ingredient.

Chemical name: Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl
IUPAC Name: Ethyl-N$^{\alpha}$-dodecanoyl-L-arginate hydrochloride
Generic names: Monohydrochloride of L-arginine N$^{\alpha}$-lauroyl-ethyl ester,
Ethyl lauroyl arginate hydrochloride,
LAE.
CAS No: 60372-77-2
Empirical formula: $C_{20}H_{41}N_4O_3Cl$
Structural formula:



Molecular weight: 421.0 g/mol

In December 2004, the Food and Drug Administration (FDA) approved lauric arginate as the common and usual name for ingredient labelling purposes. Thus, the food preservative Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl will be marketed as lauric arginate.

Chemical and Physical Properties of the Additive

Physical Properties

The physical properties of lauric arginate are reported in the following table:

Table 2: *Physical properties of lauric arginate*

Parameters analysed	Physical properties	Method
Appearance (Colour and physical state)	White powder	----
Melting temperature	50.5 to 58ºC	OECD-102; EEC-A1
Boiling temperature	Decomposes from 107ºC	OECD-103; EEC-A2
Relative density (D^{20}_4)	1.11	OECD-109; EEC-A3
Vapour pressure (at 25ºC)	$5.45 \cdot 10^{-4}$ Pa	OECD-104; EEC-A4

Parameters analysed	Physical properties	Method
Surface tension (at 19ºC)	25.43 mN/m	OECD-115; EEC-A5
Water solubility (at 20ºC)	> 247 g/kg	OECD-105; EEC-A6
Partition coefficient n-octanol/water (Log $K_{o/w}$) (at 20ºC)	1.43	OECD-107; EEC-A8
Flammability	Not flammable	EEC-A10
Explosive properties	Not explosive	EEC-A14
Relative self-ignition temperature for solids	Not self-igniting	EEC-A16
Oxidising properties	Not oxidising	EEC-A17
Particle size distribution	0% <10 μm	----
Adsorption/desorption (at 18.5ºC)	58	OECD-110

Identity

Detailed information on the identity of the active ingredient was obtained by the following techniques, for which the relevant parameters are given below (Appendix 27):

i) Ultraviolet/visible spectrum (UV/Vis),
ii) Fourier transformed infrared spectrum (FT-IR),
iii) Nuclear magnetic resonance (NMR),
iv) Electrospray mass spectrometry (ES-MS),
v) High performance liquid chromatography (HPLC) and
vi) Elemental analysis.

i) UV/Visible Spectrum:

UV/Vis (Acetonitrile/Water (85:15) v/v + 1% TFA, l =1 cm, C= 0.1083 mM)

Sample	Purity	λ (nm)	ε (dm^3/mol cm)
Q-98.250.5	95.7 %	205	2782

ii) IR Spectrum:

FT-IR (KBr disk, Q-98.250.5 purity 95.7%)

ν (cm^{-1})	Assignments
3430	-NH
3329-3173	-NH
2917-2850	C-H
1757	Ester
1648.2	C=N

1531 CONH

iii) NMR Spectrum:

^{1}H-NMR (Deuterated methanol; ^{1}H-299.9 MHz, concentration: 10 mg/700 µl, Q-98.250.5 purity 95.7%)

ppm	H	Multiplicity	Assignments	
0.9	3H	t	$C\underline{H}_3$-C	(from lauroyl group)
1.2-1.4	18H	m	C-$(C\underline{H}_2)_9$-C	(from lauroyl group)
1.6-1.8	6H	m	C-$(C\underline{H}_2)_3$-C	(from arginine group)
2.2	2H	m	$\underline{CH}_2$–C(=O)—NH–	(from lauroyl group)
3.2	3H	t	$C\underline{H}_3$-CH_2O-	(from ethyl ester group)
4.2	2H	q	CH_3-$C\underline{H}_2O$-	(from ethyl ester group)
4.4	1H	m	CH α	(from arginine group)

^{13}C-NMR (Deuterated methanol; ^{13}C-75.4 MHz; concentration: 100 mg/700 µl, Q-98.250.5 purity 95.7%)

ppm	Assignments
176.5	**C**O-NH
173.3	**C**OOEt
158.6	**C**=NH
62.3	**C**H_2-OCO
62.4	**C**H α
25.5	13 **C**H_2
14	2 **C**H_3

iv) Mass Spectrum:

ES-MS, (ESP(+) H_2O:CH_3CN (1:1) + 1% Formic, Q-98.250.5 purity 95.7%)

m/z	Assignment
385	[M-Cl^-]

v) High Performance Liquid Chromatography

HPLC (Column: Symmetry C18 5μ 150 x 3.9 mm (Waters); ACN/H_2O 50/50 v/v + 0.1% TFA; UV: 212 nm, 1000 mg/l ethyl-N^{α}-lauroyl-L-arginate HCl, Q-98.250.5 purity 95.7%, Method ID-11-1697 [Appendix 29])

RT (min)	Assignments
4.3 ± 0.07	Ethyl-N^{α}-lauroyl-L-arginate HCl

vi) Elemental Analysis

Elemental Analysis (Combustion, Q-98.250.3 purity 95.4%)

Element (%)	Theoretical[1]	Sample
C	54.73	54.84
H	9.80	9.83
N	12.77	12.71
Cl	8.06	8.31
O	14.61	14.31

Bulk chemical stability

Storage at Ambient Conditions

Chemical stability of the active ingredient of lauric arginate over time was evaluated for three samples of lauric arginate:

- LAE Batch 4497 (purity 77.8%)
- LAE Batch 5131 (purity 76.1%)
- LAE Batch 10234 (purity 88.2%)

The chemical specifications of batches 4497 and 5131 correspond to the product obtained at the end of the synthesis of lauric arginate just before the product is dried. For this reason, the specifications of these batches (reported in Table 3) do not correspond to the specification for lauric arginate proposed for use in food, the difference being due solely to the difference in water contents.

The study was carried out at room temperature and all samples were maintained in a closed container. The composition of each sample was analysed at intervals over a 1-year period for batches 4497 and 5131 and over a 2-year period for batch 10234. The parameters checked were:

- Appearance of the sample, and

[1] As ethyl-N^{α}-lauroyl-L-arginate HCl monohydrated.

- Content of the active ingredient, ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl (Appendix 30).

Table 3: *Specifications of batches 4497 and 5131*

Parameters	Specifications	Method of analysis
Aspect	White solid	Visual
Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl	71-81%	ID-11-1697
N$^{\alpha}$-lauroyl-L-arginine	≤ 2.9%	ID-11-1697
Lauric acid	≤ 3.7%	ID-11-2004
Ethyl laurate	≤ 2%	ID-11-2004

Figure 1 reports the results of stability of the active ingredient with time of the three samples studied:

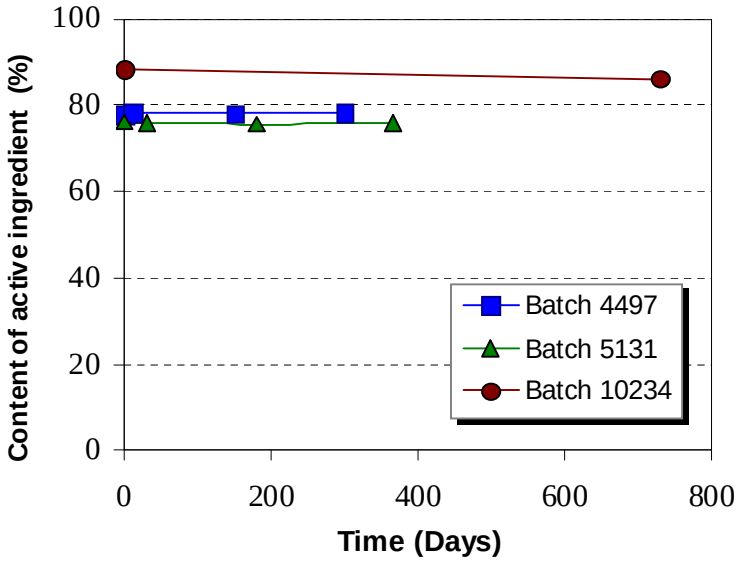


Figure 1: *Chemical stability of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl with time*

The results confirmed lauric arginate is stable under ambient storage conditions for up to two years when stored at room temperature in a closed container.

Aqueous stability of lauric arginate

The aqueous stability of lauric arginate was assessed through the determination of the extent of hydrolysis of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl under different pH conditions.

Hydrolysis is a reaction between the test substance and water that serves to mediate abiotic degradation. This reaction is particularly relevant for substances with low biodegradability since it can influence their persistence in the environment.

The aqueous stability of LAE Batch 2625 (purity 90.1%) was determined by following its hydrolysis in solutions at pH 4, 7 and 9 at a temperature of 25ºC. The results indicate that lauric arginate possesses a half-life greater than 1 year at pH 4, reducing to 57 days at pH 7 and 34 hours at pH 9 (Appendix 31).

Stability of lauric arginate at different pHs and temperatures

The stability of lauric arginate was assessed under different conditions of pH (i.e., 0.5, 1, 1.5, 2, 2.5, 3 and 3.5) and temperature (i.e., 4, 25 and 50ºC) (Appendix 32).

Lauric arginate is a powdered substance but is supplied for commercial use dissolved in a suitable carrier.

To evaluate stability of the commercial product, Mirenat-N Batch 5587 (19.3% solution of ethyl-N^{α}-lauroyl-L-arginate HCl in propylene glycol) was used. Testing was done in the presence of citric, phosphoric, tartaric, malic or fumaric acids for time periods up to 50 days.

Approximately every 10 days, samples were analysed to determine the content of ethyl-N^{α}-lauroyl-L-arginate HCl (to determine the extent of hydrolysis).

The results of this study showed that at temperatures above 25ºC and at pH between 2 and 3, significant degradation of the active ingredient of lauric arginate can occur. Therefore, the results of this study suggested that the effect of temperature combined with pH markedly influences the hydrolysis of ethyl-N^{α}-lauroyl-L-arginate HCl. At low pH and at room temperature (i.e., 25ºC), the molecule is relatively stable.

The study also demonstrated that exposure to fumaric acid yielded the least amount of hydrolysis in comparison with the other acids studied at pH 2 and 50ºC.

It was concluded that the combination of high temperature (i.e., 50ºC) and low pH (i.e., 2 or 3) can result in significantly greater hydrolysis.

The results of this study were particularly useful for establishing the range of conditions of use appropriate for lauric arginate.

The results indicate that lauric arginate should not be used in applications that combine very low pH (e.g., < 3) with high temperatures (e.g., 50ºC) for extended periods of time (e.g., more than 10 to 20 days). However, it was also concluded that the proposed uses of this product make it highly unlikely that such conditions would be experienced in practical use. Consequently, it could be concluded that stability is not an issue under the proposed conditions of use.

Impurity Profile

The content of the active ingredient of lauric arginate is between 85 to 95%. The composition of lauric arginate is provided in the table below:

Lauric arginate composition

Composition	Range w/w (%)	CAS No.	Method[1]
Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl	85-95	60372-77-2	ID-11-1697
N$^{\alpha}$-lauroyl-L-arginine	≤ 3	42492-22-8	ID-11-1697
Lauric acid	≤ 5	143-07-7	ID-11-2004
Ethyl laurate	≤ 3	106-33-2	ID-11-2004
L-arginine HCl	≤ 1	1119-34-2	ID-11-2465
Ethyl arginate 2HCl	≤ 1	36589-29-4	ID-11-2465
Ash	≤ 2	---	ID-11-083
Water	≤ 5	7732-18-5	ID-11-020

Minor by-products present in lauric arginate

The main impurities present in lauric arginate are:

- LAS,
- Lauric acid,
- Ethyl laurate,
- L-arginine HCl and
- Ethyl arginate 2HCl.

HPLC analysis revealed three additional peaks (P2, P3 and P4)[2] that are considered to be minor impurities.

In agreement with HPLC-MS studies, the by-products P2 and P4 share their basic structure with that of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl but they both possess a second lauroyl group condensed with the guanidium group of arginine and in the case of the by-product P2, the ester group is replaced by a carboxylic acid group. Quantification of these by-products, on the basis of the intensity of the protons identified as belonging to P2 and P4, indicates their content is less than 1% and 0.5% respectively. The ^{13}C satellite signals corresponding to the methyl component of the lauroyl group also indicate that impurities P2 and P4 are present in a proportion smaller than 1% and 0.5% respectively in a sample of LAE Batch 10234 (purity 88.2%) (Appendices 33, 34).

In the case of by-product P3, high-resolution MS data confirm that the compound has the same structure as that of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl but with an extra lauroyl group condensed with the amine group of arginine. A quantification study of P3 demonstrated that content of this by-product in a sample of LAE Batch 12547 [purity 91.87%][3] was 0.064% (Appendices 35, 36).

Quantification of these trace impurities is not included in the specification of lauric arginate because their presence is insignificant and their peaks are controlled to be consistently and reproducibly visualised by HPLC when historical data from analyses of lauric arginate batches are compared. They are reported in this dossier for the sake of completeness.

[1] The method corresponds to those described in Appendices 37-39.
[2] The peak designated as P1 was identified as lauric acid and it is considered one of the main by-products of lauric arginate.
[3] The original report states by error a purity of 89.1%.

Manufacturing Process

The manufacturing process is described in Confidential Appendix 31.

Specifications for Identity and Purity

Name:	Lauric arginate
Synonyms:	LAE Monohydrochloride of L-arginine N^{α}-lauroyl-ethyl ester Ethyl lauroyl arginate hydrochloride
Definition:	
Chemical name	Ethyl-N^{α}-lauroyl–L-arginate HCl
ELINCS	434-630-6
Chemical formula	$C_{20}H_{41}N_4O_3Cl$
Molecular weight	421.02
Assay of ethyl-N^{α}-lauroyl–L-arginate HCl	Not less than 85% and not more than 95%
Description	White powder
Identification:	
Solubility	Soluble in deionised water, propylene glycol, glycerine and ethanol
pH of 1% solution	Not less than 3 and not more than 5
Purity:	
N^{α}-lauroyl–L-arginine	Not more than 3%
Lauric acid	Not more than 5%
Ethyl laurate	Not more than 3%
L-arginine HCl	Not more than 1%
Ethyl arginate 2HCl	Not more than 1%
Ash	Not more than 2%
Water	Not more than 5%
Ethanol	Not more than 0.2%
Arsenic	Not more than 3 mg/kg
Cadmium	Not more than 1 mg/kg
Lead	Not more than 1 mg/kg
Mercury	Not more than 1 mg/kg

Information for Food Labelling

The food additive is a preservative with the common name lauric arginate. No code number has been assigned to this food additive.

Analytical Method for Detection in food

Introduction

Lauric arginate is proposed to be used as a food preservative in the following food categories:

- Flavoured water and high energy drinks and soft drinks
- Fruit juices and drinks
- Tea
- Meat products
- Cheese including soft/cream/processed and mozzarella
- Prepared salads
- Fruit preparations used in fruit desserts
- Savoury toppings or fillings- essentially sauces such as tomato paste used in ready to eat pizzas, etc.
- Dehydrated legumes
- Fish products
- Low joule jams
- Confectionery products (e.g. chewing gums)
- Soups
- Miscellaneous

The content of the active ingredient of lauric arginate present in food matrices is quantified by reverse-phase, high-performance liquid chromatography (RP-HPLC). The proposed method involves the use of different sample preparation techniques depending on the type of food matrix that is to be analysed. Two of the techniques, *Method A* and *Method B,* are suitable for solid and semi-solid food matrices (method ID-11-2493) (Appendix 40). A third technique, *Method C* is suitable for liquid food matrices (method ID-11-2494) (Appendix 41).

Both procedures use the same equipment, reagents and chromatographic conditions; they differ only in the way to prepare the sample.

Once ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl has been extracted, it is analysed by reversed phase high performance liquid chromatography (RP-HPLC) and quantified using an external standard curve. Either isocratic or gradient chromatographic conditions can be employed. However, based on the analysis of several samples using both isocratic and gradient chromatographic conditions, it was determined that gradient conditions were more suitable for the quantification of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl from a complex food matrix where the presence of chromatographic interferences can affect the analysis.

LAS, which comes from the hydrolysis of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl, is also detected using Methods A, B and C. Although quantification of LAS is possible, it is not the goal of this analysis.

Examples of solid and semi-solid food matrices from which ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl has been successfully extracted[4]:

- Cooked meat products: cooked ham, Frankfurt and Bratwurst sausages, nuggets

[4] The extraction of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl has been successfully studied in a wide variety of foods, however the intended uses of lauric arginate as food preservative consider only a reduced number of these food categories.

- Raw meat products: hamburgers, ground pork, minced meat, steak, fresh sausage, chicken breasts
- Meat toppings for pizza
- Cold crabmeat
- Tripe in sauce
- Chicken skin
- Rice
- Bechamel sauce (butter, flour and milk)
- Chickpeas
- Fried tomato sauce

Examples of liquid food matrices from which ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl has been successfully extracted[1]:

- Pineapple juice
- Coffee liquor
- Liquefied egg product
- Orgeat (a cold drink made of tiger nuts)
- Juice concentrates
- Soft drinks: carbonated flavoured drinks, non carbonated flavoured drinks, sports drinks

Equipment and reagents[5]

- Extraction thimbles, 30 mm x 80 mm
- Chloroform, analysis grade
- Methanol, analysis grade
- Analytical balance, MC1, precision ≥ ± 0.1 mg
- Centromix Centrifuge, ≥ 4,000 rpm
- High Performance Liquid Chromatograph
- Column: Symmetry® C_{18} 5μm 150 mm x 3.9 mm
- Acetonitrile (ACN), HPLC grade
- Water MilliQ®
- Trifluoroacetic acid (TFA), synthesis grade
- Volumetric flasks and measuring pipettes, A class
- Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl standard: Q-98.250 purity ≥95%
- LAS standard: Q-98.251 purity ≥98%
- Nylon filters, 0.45 μm
- Magnetic stirring rods
- Homogenizer
- Stomaker
- Bag filters for Stomaker

Chromatographic conditions

Isocratic chromatographic conditions
Column: Symmetry® C_{18} 5μm 150 x 3.9 mm
Solvent: ACN/H_2O (50/50 v/v)+ 0.1% TFA[6]
Flow rate: 1 ml/min
Wavelength: 215 nm (range: 190-300 nm)

[5] Other comparable equipment and reagents can be used for this analysis.
[6] 500 ml of H_2O + 500 ml of ACN + 1 ml TFA.

Injection volume: 10 μl
Retention time for ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl: 4.3 minutes (± 0.2)[7]
Retention time LAS: 2.3 minutes (± 0.2)[7]
Total time: 10 minutes

Gradient chromatographic conditions

- Column: Symmetry® C_{18} 5 μm 150 x 3.9 mm
- Solvent A: H_2O + 0.045 % TFA[8]
- Solvent B: ACN + 0.036 % TFA[9]
- Wavelength: 215 nm
- Injection volume: 20 μl
- Retention time ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl: 19 minutes (± 0.3)[10]
- Retention time LAS: 16.2 minutes (± 0.3)[10]
- Total time: 40 minutes (32 minutes of gradient + 8 minutes of equilibration)

Experimental conditions

Time (min)	Flow Rate (ml/min)	Mobile Phase	
		% A	% B
0	1.0	70	30
5	1.0	70	30
25	1.0	30	70
27	1.3	0	100
30	1.3	0	100
32	1.0	70	30

Procedure

Preparation of standard solutions

Standard ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and LAS solutions are prepared by adding powdered substrate to ACN/H_2O (50/50 v/v + 0.1% TFA). The range of substrate concentrations used to create a standard curve must bracket the substrate concentration expected to be present in the analyzed food sample. At least four different substrate concentrations ("standards") must be prepared and analyzed to create the standard curve.

Sample Preparation[11]

Due to unpredictable chromatographic interferences from new food matrices treated with ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl, the availability of different methods of sample preparation

[7] The retention times for ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and LAS were obtained at LAMIRSA laboratories under the reported chromatographic conditions.
[8] 1 L H_2O + 450 μl TFA.
[9] 1 L ACN + 360 μl TFA.
[10] The retention times for ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and LAS were obtained at LAMIRSA laboratories under the reported chromatographic conditions.
[11] The values of mass and volume reported in this document are only indicatives and suitable for the analysis of a food matrix with an approximate concentration of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl equal to 77 mg/kg. Mass, volumes and conditions used for analysis may need to be adjusted for each new case.

is required. It is difficult to determine *a priori* which sample preparation method will completely extract residual ethyl-N^{α}-lauroyl-L-arginate HCl from a new food matrix to be analysed. In order to make this determination, a known concentration of ethyl-N^{α}-lauroyl-L-arginate HCl is added to a sample of the food matrix of interest. Both methods A and B are employed for the extraction of ethyl-N^{α}-lauroyl-L-arginate HCl and the method which maximally recovers the known quantity of the active ingredient is determined.

The suggested mass and volume values presented in this analytical method are appropriate for extracted ethyl-N^{α}-lauroyl-L-arginate HCl concentrations in the range of 10 to 1,000 mg/l.

Method A: direct analysis from a solid and semi-solid food matrix

A representative sample of food is homogenized using a food grinder. Approximately 20 ml of ACN + 0.1% TFA is added to approximately 12 g of the ground sample. In a closed container[12], at room temperature and in the dark, the sample and solvent are stirred for approximately 17 hours using a magnetic stirring bar.

Another technique is to ground and homogenise the sample using a Stomaker. In this case, the amount of sample used is between 10 and 20 g and the solvent volume is between 50 and 100 ml of ACN + 0.1% TFA. The sample and the solvent are grounded for 9 minutes using the Stomaker.

For food matrices with low water content is more suitable to employ ACN/H_2O (50/50 v/v) + 0.1% TFA.

Using a plastic syringe, an aliquot of approximately 5 ml is removed from the liquid fraction and then filtered through a 0.45 μm nylon filter. This filtered sample is then analysed using the appropriate chromatographic method.

Method A special cases: food matrices which are difficult to stir

Mixing certain food matrices with the magnetic stir bar may be difficult.
In these cases, it is better to perform the extraction using a sonicator. Two examples and sonication method parameters are shown in the table below.

Table 4: *Sonication conditions according to the type of matrix*

Food Matrix	***Sample Mass (g)***	**Solvent[13] Volume (ml)**	***Sonication Conditions***
Tripe in sauce	3-5	10	60 minutes at 35ºC
Chickpeas	10-20	50	2-4 hours at room temperature

Once the extraction process is completed, approximately 5 ml of the liquid fraction is collected with a plastic syringe, filtered through a 0.45 μm nylon filter, and then analysed using the appropriate chromatographic method.

[12] The extraction process must be performed in a closed container in order to avoid the loss of solvent by evaporation.
[13] Solvent: ACN/H_2O (50/50 v/v) + 0.1% TFA.

Method B: extraction from a solid food matrix by Soxhlet[14]

Remove all water from the sample by means of lyophilisation using the following procedure.

Homogenize a representative sample of the food to be analysed. Weigh the homogenized sample into a tared plastic bag and spread it well to maximize its surface area. Freeze the sample at -15ºC (or colder) for at least 8 hours at 0.075 to 0.750 torr. The lyophilisation process is complete when the weight of the sample is constant for two consecutive days.

Note: It is important to determine the weight of the sample before and after lyophilisation.

Once the sample has been lyophilised, mix it very well and place approximately 12 g of the sample inside a 30 mm x 80 mm extraction thimble. The extraction process is performed in a Soxhlet apparatus using approximately 175 ml of a chloroform/methanol mixture, (80/20 v/v) for 8 hours. The extract obtained is then concentrated in a rotary evaporator at approximately 14 torr. The extract is mixed with approximately 10 ml of ACN/H_2O (50/50 v/v) + 0.1% TFA. This suspension is then centrifuged at 4,000 rpm for 20 minutes in order to separate the hydrophobic phase from the hydrophilic phase. The hydrophobic phase is discarded and the hydrophilic phase, which is practically transparent, is filtered through a 0.45 μm nylon filter. The filtered sample is then analysed using the appropriate chromatographic method.

Method C: analysis from a liquid food matrix

Weigh 5 to 20 g of sample into a volumetric flask of 25-50 ml and make up to this volume with ACN + 0.1% TFA or ACN/H_2O (50/50 v/v) + 0.1% TFA and mix well (see examples in the table 5.8)[15]. Approximately 5 ml are filtered through a 0.45 μm nylon filter. The filtered sample is analysed using the appropriate chromatographic method.

Table 5*: Conditions for extraction of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl from liquid food matrices*

Food matrix	Sample mass (g)	Stirring time (min)	Final volume (ml)
Pineapple juice	10-20	--	25-50
Fruit concentrates	5	5	25
Carbonated flavoured drinks	10-20	10	25-50
Non carbonated flavoured drinks	10-20	10	25-50
Sports drinks	10-20	10	25-50
Coffee liquor	10-20	--	25-50
Orgeat	6-12	20	50

[14] The values of mass and volume reported in this document are only indicative and suitable for the analysis of a food matrix with an approximate concentration of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl equal to 77 mg/Kg. Mass, volumes and conditions used for analysis may need to be adjusted for each new case.

[15] The weight of the sample, volume of the solvent and duration of the mixing will depend on the type of food matrix and the expected concentration of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl to be extracted from the food matrix.

Quantification

A standard curve is created with at least four standard concentrations bracketing the expected ethyl-N^{α}-lauroyl-L-arginate HCl concentration in the food matrix and plotting standard concentration *versus* peak area. Chromatographic conditions are optimised by means of the instructions from the chromatographic software. The standard curve must have a correlation coefficient (r^2) >0.998 and the slope must not be different from that derived from a minimum of two standard curves prepared under the same conditions with a percent relative standard deviation (% RSD) <2. If these two conditions are not fulfilled, then the standard curve must be discarded and a new standard curve with freshly prepared standards must be created.

The concentration of residual ethyl-N^{α}-lauroyl-L-arginate HCl in the analysed food is calculated depending on the method of analysis used.

Methods A and C: Ethyl-N^{α}- lauroyl-L-arginate HCl Quantification

$$C = \frac{C_{obt}\ x\ V}{W}$$

Where,

C: Concentration of residual ethyl-N^{α}-lauroyl-L-arginate HCl found in the analysed food (mg/kg)
C_{obt}: Concentration of ethyl-N^{α}-lauroyl-L-arginate HCl in the analysed phase (mg/l)
V: Total volume of solvent used for extraction (l)
W: Mass of the treated sample (kg)

Method B: Ethyl-N^{α}-lauroyl-L-arginate HCl quantification

$$C = \frac{C_{obt}\ \ x\ V\ x\ W_2}{W_1\ x\ \ W_3}$$

Where,

C: Concentration of residual ethyl-N^{α}-lauroyl-L-arginate HCl found in the analysed food (mg/kg)
C_{obt}: Concentration of ethyl-N^{α}-lauroyl-L-arginate HCl in the analysed phase (mg/l)
V: Total volume of solvent used for extraction (l)
W_1: Mass of aliquot of the extracted lyophilised sample (kg)
W_2: Mass of the sample after lyophilisation (kg)
W_3: Mass of the wet sample (kg)

Note: Any additional dilutions employed must be included in the final quantification calculation.

Technical note

Analysis of food samples with a high fat content, such as pâté, must not be performed using the methods presented here because the chromatographic column can be irreversibly damaged by fatty acids. In this case, a pre-column should be employed in order to eliminate the impurities.

Chromatograms of standards

Isocratic conditions

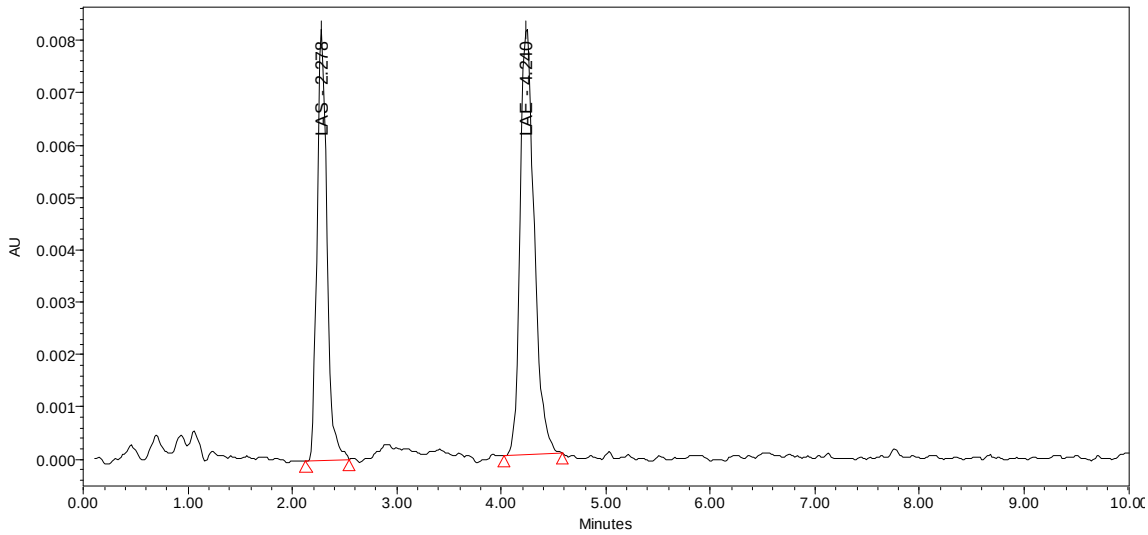


Figure 2: *Detection of 50 ppm of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl[12] and 20 ppm of LAS*

Gradient conditions

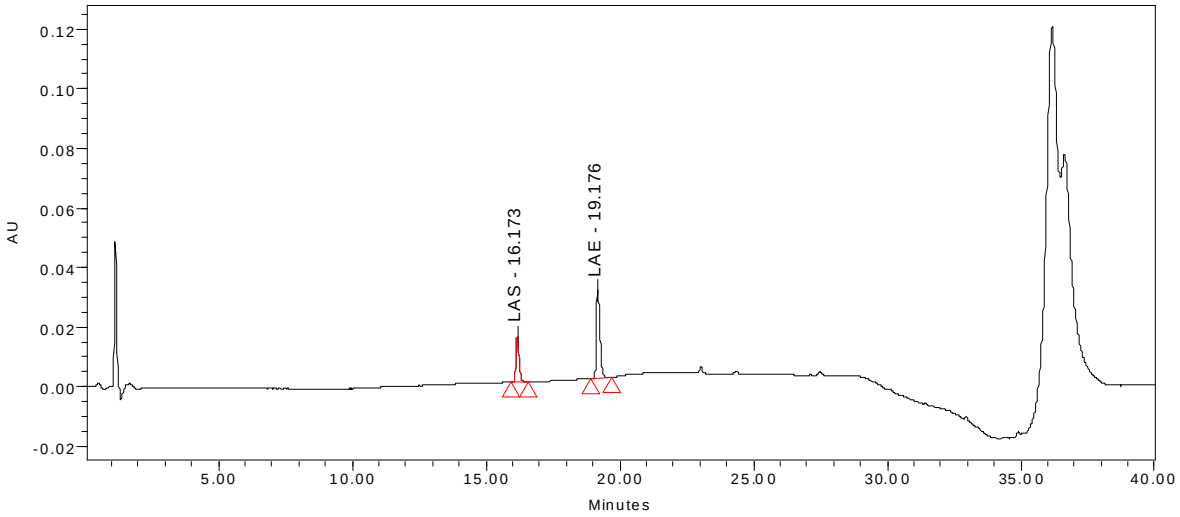
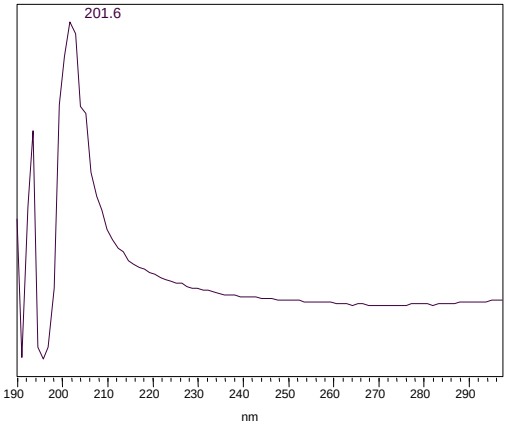


Figure 3: *190 ppm of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl[16] and 100 ppm of LAS*

[16] Marked as LAE on the chromatogram.

LAS



Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl

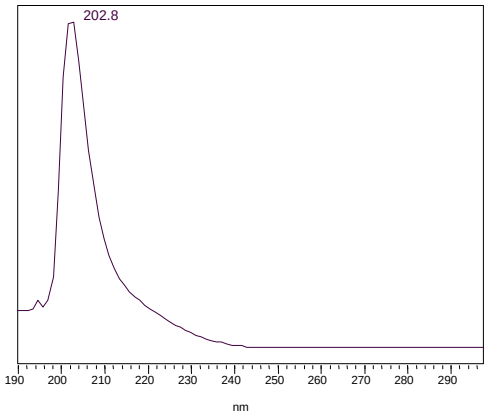


Figure 4: *Ultraviolet profiles of LAS and ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl*

ANALYTICAL METHOD FOR LAURIC ARGINATE AND ITS BY-PRODUCTS

Analysis of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and N$^{\alpha}$-lauroyl-L-arginine

The quantitative determination of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and N$^{\alpha}$-lauroyl-L-arginine (LAS) is carried out by high performance liquid chromatography (HPLC) using the following chromatographic conditions (ID-11-1697) (Appendix 42):

- Column: Symmetry C_{18} 5 μm 150 x 3.9 mm
- Flow rate: 1 ml/min
- Injection volume: 10 μl
- Wavelength: 215 nm
- Mobile phase: ACN/water 50/50 v/v + 0.1% TFA
- Retention time[17]: LAS 2.2 min (± 0.03) and ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl 4.3 min (± 0.07)

Reagents employed:
- Trifluoroacetic acid (TFA)
- Acetonitrile (HPLC or multisolvent grade)
- Water (MilliQ grade)

Standards:
- Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl: Q-98.250 purity >95%
- LAS: Q-98.251 purity >98%

Stock solutions of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and LAS:
Two solutions are prepared: solution A and solution B. Solution A is prepared by weighing approximately 25 mg (± 0.1) of LAS standard into a 25 ml volumetric flask and diluting to volume with mobile phase. The final concentration of LAS is about 1000 mg/l.

Solution B is prepared by weighing approximately 150 mg (± 0.1) of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl standard into a 50 ml volumetric flask and dissolving it with a few millilitres of the mobile phase. Then, 5 ml of solution A is added and the whole diluted to volume with mobile phase. The concentration of this solution is approximately 100 mg/l of LAS and 3000 mg/l of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl.

Standard solutions for the calibration curve:
Volumes of 4, 6, 10 and 15 ml of solution B are each diluted to a volume of 50 ml with mobile phase. The final concentration of each standard solution is approximately:

Volume Solution B (ml)	Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl (mg/l)	LAS (mg/l)
4	240	8
6	360	12
10	600	20
15	900	30

[17] The retention times for ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and LAS were obtained at LAMIRSA laboratories under the reported chromatographic conditions.

Calibration curve:

Once the chromatographic system is equilibrated, a volume of 10 µl of each standard solution is injected in order to make the calibration curve.

Sample preparation and quantitative analysis:

The sample size must be such that after injection of 10 µl of test solution both analytes are found in the middle of the calibration curve. The sample solution is prepared using a 50 ml volumetric flask and the mobile phase as solvent. At least three replicates should be analysed.

The percentage of each compound is calculated as follows:

$$\%\,LAS = \frac{C_{LAS\,Sample}}{C_{Sample}} \times 100$$

Where,

$C_{LAE\ Sample}$: concentration of ethyl-N^{α}-lauroyl-L-arginate HCl found in the sample solution (mg/l)

$C_{LAS\ Sample}$: concentration of LAS found in the sample solution (mg/l)

$C_{\ Sample}$: concentration of test sample (mg/l)

<u>Analysis of lauric acid and ethyl laurate</u>

The quantitative determination of lauric acid (LOH) and ethyl laurate (LOEt) is carried out by high performance liquid chromatography (HPLC) using the following chromatographic conditions (ID-11-2004) (Appendix 38):

- Column: Symmetry C_{18} 5 µm 150 x 3.9 mm
- Flow rate: 1 ml/min
- Injection volume: 10 µl
- Wavelength: 212 nm
- Mobile phase: ACN/water 85/15 v/v + 0.1% TFA
- Retention time[18]: LOH 3.7 min (± 0.12) and LOEt 11.2 min (± 0.46)

<u>Reagent employed</u>:

- Trifluoroacetic acid (TFA)
- Acetonitrile (HPLC or multisolvent grade) % Ethyl-N^{α}-lauroyl-L-arginate HCl
- Water (MilliQ grade)

<u>Standards</u>:

- LOH: Q-98.257 purity >99%
- LOEt: Q-98.258 purity >99%

[18] The retention times for LOH and LOEt were obtained at LAMIRSA laboratories under the reported chromatographic conditions.

Stock solution of LOH and LOEt:

A stock solution is prepared by weighing approximately 250 mg (± 0.1) of LOH standard into a 50 ml volumetric flask and dissolving it in a few millilitres of mobile phase. To this is added approximately 75 mg (± 0.1) of LOEt standard. The flask is shaken and the contents are diluted to volume with the mobile phase. The final concentration of this solution is approximately 5000 mg/l LOH and 1500 mg/l of LOEt.

Standard solutions for the calibration curve:

Volumes of 3, 5, 7 and 10 ml of stock solution are each diluted to 50 ml with the mobile phase. The final concentration of each standard solution is approximately:

Volume (ml)	LOH (mg/l)	LOEt (mg/l)
3	300	90
5	500	150
7	700	210
10	1000	300

Calibration curve:

Once the chromatographic system is equilibrated, a volume of 10 µl of each standard solution is injected in order to make the calibration curve.

Sample preparation and quantitative analysis:

The sample size must be such that after injection of 10 µl both analytes are found in the middle of the calibration curve. The sample solution is prepared using a 50 ml volumetric flask and the mobile phase as solvent. At least three replicates should be analysed.

The percentage of each compound is calculated as follows:

$$\%LOH = \frac{C_{LOH\ Sample}}{C_{Sample}} \times 100$$

$$\%LOEt = \frac{C_{LOEt\ Sample}}{C_{Sample}} \times 100$$

Where,

$C_{LOH\ Sample}$: concentration of LOH found in the sample solution (mg/l)
$C_{LOEt\ Sample}$: concentration of LOEt found in the sample solution (mg/l)
C_{Sample}: concentration of test sample (mg/l)

Analysis of L-arginine HCl and ethyl arginate 2HCl

The quantitative determination of L-arginine HCl and ethyl arginate 2HCl is carried out by high performance liquid chromatography (HPLC) using the following chromatographic conditions (ID-11-2465) (Appendix 43):

- Column: Waters μBondepack, C_{18} 10 μ 300 x 3.9 mm
- Flow rate: 0.8 ml/min
- Injection volume: 10 μl
- Wavelength: 340 nm
- Mobile phase: sodium heptanesulphonate: phosphoric acid: sodium dihydrogen phosphate: methanol (1:1:1:1.5)
- Ionic pair technique using sodium heptanesulphonate in a buffered medium of phosphoric/sodium phosphate/methanol
- Post-column derivatization at alkaline pH in presence of orthophtaldehyde/2-mercaptoethanol at 65ºC in a tubular reactor of teflon

Reagent employed:
- Methanol (HPLC or multisolvent grade)
- Water (MilliQ)
- Sodium heptanesulphonate monohydrate (HPLC grade)
- Boric acid
- Phosphoric acid, 85%
- Sodium dihydrogenphosphate
- Orthophtaldialdehyde
- 2-Mercaptoethanol

Standards:
- L-arginine HCl: Q-98.274 purity ≥99%
- Ethyl arginate 2HCl: Q-98.275 purity ≥99%

Stock solutions of L-arginine HCl and ethyl arginate 2HCl:

Two solutions are prepared: solution A and solution B. Solution A contains 40 mg L-arginine HCl dissolved in water MilliQ and volumetrically diluted to a final volume of 100 ml. Solution B contains 190 mg of ethyl arginate 2HCl dissolved in water MilliQ and volumetrically diluted to a final volume of 25 ml.

Standard solutions:

Standard solutions are prepared independently by mixing volumes of stock solutions A or B in accordance with the following table and making each one up to 25 ml with the mobile phase. The final concentrations of L-arginine HCl and ethyl arginate 2HCl in the standard solutions are given in the following table.

Volume solution A (ml)	L-arginine HCl (mg/l)	Volume solution B (ml)	Ethyl arginate 2HCl (mg/l)
1	16	1	300
2	32	2	600
3	48	3	900
4	64	4	1200

Calibration curve:

Once the chromatographic system is equilibrated, a volume of 10 µl of each standard solution is injected in order to make the calibration curve.

Sample preparation and quantitative analysis:

The sample size must be such that after injection of 10 µl both analytes are found in the middle of the calibration curve. The sample solution is prepared using a 25 ml volumetric flask and the eluent or water MilliQ as solvent.

The percentage of each compound is calculated as follows:

$$\%\, L-Arginine\ HCl = \frac{C_{Arginine\ Sample}}{C_{Sample}} \times 100$$

$$\%\, Ethyl\ \ arginate\ 2HCl = \frac{C_{Ethyl\ arginate\ 2HCl\ Sample}}{C_{Sample}} \times 100$$

Where,

$C_{Arginine\ Sample}$: concentration of L-arginine HCl found in the sample solution (mg/l)
$C_{Ethyl\ arginate\ 2HCl\ Sample}$: concentration of ethyl arginate 2HCl found in the sample solution (mg/l)
C_{Sample}: concentration of sample (mg/l)

Validation of methods

Methods ID-11-1697 (Appendix 42) and ID-11-2004 (Appendix 38), which are employed to determine the content of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and its three main by-products, have been validated by NOTOX B.V. according to the OECD Principles of GLP (Appendix 44).

In order to ensure that the methods and the results obtained reflected the raw data, a sample of LAE Batch 4272 (purity 87.9%) was analysed and the specificity, linearity, precision (repeatability), limit of detection and limit of quantification, associated with the measurement of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl, LAS, LOH and LOEt were determined.

Test substance:

LAE Batch 4272	**% (w/w)**
Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl	87.9
LAS	1.68
Lauric acid	3.05
Ethyl laurate	0.99

The following table summarises the results obtained:

Table 6*: Summary of results obtained according to linearity, precision, limit of detection and limit of quantification. Analytes determined: ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl, LAS, LOH and LOEt*

	Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl	**LAS**	**LOH**	**LOEt**
Specificity	No interference peaks from impurities were observed in the chromatograms of the samples			
Linearity ***Range:*** ***Slope:*** ***Intercept:*** ***R:***	 59-886 mg/l 602 234 0.999992	 2-30 mg/l 575 3.78 0.9998	 101-1210 mg/l 84.6 -650 0.99996	 35.9-431 mg/l 120 -1.05 10^3 0.99992
Precision ***Mean content*** ***(SD)***	89.1% w/w[19] (0.3)	1.97% w/w (0.02)	3.10% w/w (0.06)	0.90% w/w (0.02)
Limit of detection[20]	0.9 mg/l	0.6 mg/l	9.7 mg/l	18 mg/l
Limit of quantification	62.0 mg/l	2.0 mg/l	101 mg/l	35.9 mg/l

The validity of methods ID-11-1697 and ID-11-2004 is confirmed by the fact that the results reported in the table above reflect the real composition of the sample analysed LAE Batch 4272 with an acceptable standard deviation.

[19] NOTOX expressed LAE Batch 4272 as 92.9% ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl·H_2O. The results reported in this table are re-calculated in order to be expressed as ethyl- N$^{\alpha}$-lauroyl-L-arginate HCl (without the molecule of H_2O).
[20] The limit of detection was determined at an injection volume of 10 µl.

Information Related to Safety of the Food Additive

Toxicokinetics and Metabolism

Studies using a mixture of unlabelled lauric arginate and ^{14}C-ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl (arginine portion of the molecule uniformly radiolabelled) show that once ingested lauric arginate is well absorbed, rapidly metabolised, incorporated into naturally occurring products via the urea and citric acid cycles, distributed to liver, and slowly excreted as carbon dioxide via the expired air, and to a very low extent via the urine and faeces.

Two studies were performed in rats to investigate how lauric arginate is metabolised. The test substance was a mixture of unlabelled lauric arginate and ^{14}C-ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl (arginine portion of the molecule uniformly radiolabelled). This mixture was referred to as ^{14}C-LAE. In these studies the absorption, distribution and excretion rate of radioactivity were determined *in vivo* after a single oral dose. In the first study (Appendix 17) the metabolism, excretion and retention of radioactivity of ^{14}C-LAE was evaluated *in vivo*. In the second study (Appendix 18), which complemented the first, the metabolism of lauric arginate was extensively studied *in vitro* using S-9 liver fractions and plasma extracts, and *in vivo* using 6 male rats and analysing the metabolites present in plasma.

A third study was conducted to obtain further information on the plasma pharmacokinetics of lauric arginate and its metabolite (N$^{\alpha}$-lauroyl-L-arginine, LAS) in rats after a range of oral doses administered once in different formulations (Appendix 23). Blood samples were taken up to 8 hours post-dose and plasma concentrations of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl (the active ingredient of lauric arginate) and LAS were measured and pharmacokinetic parameters calculated.

Metabolism in the rat: determination of absorption and excretion rates of ^{14}C-LAE
(Study LMA 017/983416, Huntingdon Life Science, Huntingdon, UK, 1998).

Introduction:

The objectives were to assess absorption and rates and routes of excretion of radioactivity after a single oral dose of ^{14}C-LAE to male rats and to obtain information on the biotransformation of ^{14}C-LAE in rats (Appendix 17).

Study Design:

^{14}C-LAE was administered orally at a nominal dose of 180 mg/kg bw to 4 male rats. The rats were sacrificed 120 hours later. Excreta samples and exhaled carbon dioxide were collected at intervals throughout the study.

Good Laboratory Practice:

The study was conducted in compliance with the EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal Management:

Male Sprague-Dawley (Crl:CD®(SD)BR) rats obtained from Charles River, Margate, Kent, UK were used. After an acclimatisation period of 7 days, 4 animals were transferred to single housing in glass metabowls with urine/faeces separators (overnight pre-dose to the end of the study). Food and water were available *ad libitum*. Room temperature was 19-25ºC, relative humidity 40 to 70% and a 12 hour artificial light/dark cycle was maintained. Animals were aged 7-9 weeks and were within the bodyweight range of 222-240 g on dosing.

Test substance:
The test substance was a mixture of non-radiolabelled LAE [NB - Batch 5159 (purity 69.1%[21])], dissolved in a solution of radiolabelled ethyl-N^{α}-lauroyl-L-arginate HCl (Batch NPE/LMA00165, purity >97%) in which the arginine portion of the molecule was uniformly labelled with ^{14}C. The final volume of the test substance was adjusted so that the ^{14}C-LAE concentration was 64.7 mg/ml and the specific activity was 0.38 μCi/mg (0.014 MBq/mg). The radiochemical purity was 99.4%.

Test substance formulation:
Formulated ^{14}C-LAE, was prepared on the day of dosing. A known volume of the test substance was concentrated and thoroughly mixed with 1% aqueous methylcellulose (the vehicle). The nominal concentration of this formulation was 80 mg ^{14}C-LAE /ml with a radiochemical purity higher than 97%.

Administration of the test substance and dose level:
The test substance formulation was administered as a single bolus dose by gastric intubation at a dose volume of 2.5 ml/kg bw to give a target nominal dose of 200 mg/kg bw. Aliquots of the dosing suspensions were radioassayed and the actual dose calculated for each rat was 177-180 mg ^{14}C-LAE/kg bw.

Sample collection:
After dosing, urine, expired air and faeces were collected separately from each animal at intervals for 5 days. Urine and expired air samples were collected at 0-8, 8-24 hours and then at 24 hour intervals. Samples of faeces and cage washings were collected at 24 hour intervals. Faeces and urine samples were collected into cooled receivers and the expired air was trapped in an appropriate solvent.

One hundred and twenty hours (5 days) after dosing each rat was sacrificed and the gastrointestinal tract (GIT), including contents, liver and the remaining carcass were analysed.

Analytical methods employed to identify radioactive components:
The absorption and rates and routes of excretion of radioactivity were assessed and information on the biotransformation of ^{14}C-LAE obtained using Liquid Scintillation Counting (LSC), High Performance Liquid Chromatography (HPLC) and Thin-Layer Chromatography (TLC).

Results:

Excretion and retention of radioactivity:
Five days after dosing, the mean total recovery of radioactivity was 99.5% of the administered dose. In general, the pattern and rate of excretion by individual animals was very similar.

The major route of excretion was as carbon dioxide. A mean total of 23.9% of the dose was eliminated via this route in the 24 hours following dosing, increasing to a mean total of 36.6% of the dose over 5 days. Excretion in urine and faeces was low; a mean total of 11.8% and 4.3% of the dose respectively was excreted over the 5 day collection period. The low % in faeces indicated that a high percentage of the test substance was completely absorbed. The largest proportion of radioactivity was recovered from the carcass, a mean total of 41.0% of the dose, of which 2.0% was

[21] The purity of this batch does not correspond to the specification for lauric arginate. LAE Batch 5159 was obtained following the same synthesis process described in section 6.1, but using a different filter press. The only difference from lauric arginate as specified is the water content.

measured in the GIT (45.8 μg equivalent/g tissue net weight of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl) and 3.4% of the dose in the liver (120 μg equivalent/g tissue net weight ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl).

The results are summarised in Figure 5:

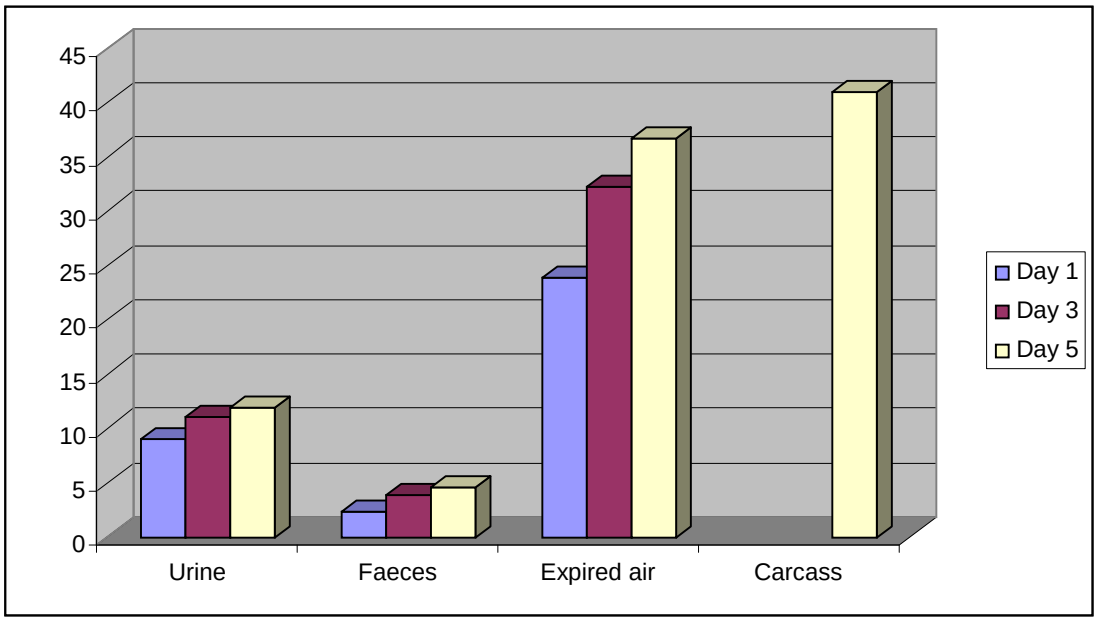


Figure 5: *Percentage of radioactive dose detected in samples of urine, faeces, expired air and carcass at various time points*

Quantification and identification of radioactive components in urine:
Analysis of the urine between 0 to 24 hours by HPLC showed a single peak with a retention time of 2 minutes. This peak represented a mean 9% of the administered dose. However, urine was also analysed by Thin Layer Co-chromatography in a solvent system, which separated up to 7 radioactive components. The major component represented a mean 7.7% of the dose and was found to co-chromatograph with ^{14}C urea. All the other components present represented ≤0.2% of the dose and were more polar than the major component. No radioactivity with the retention time of unchanged ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl was detected.

Conclusions:

After oral administration of 180 mg ^{14}C-LAE/kg bw, radioactivity was well absorbed and slowly eliminated. Five days after dosing a mean total of 41.0% of the dose was retained in the carcass including the liver and GIT.

The main route of elimination was as carbon dioxide in the expired air, a mean total of 36.6% of the dose was eliminated by day 5. Excretion in urine and faeces was low, approximately 11.8% and 4.3% respectively by Day 5.

Analysis of urine showed that the major component present co-chromatographed with ^{14}C-urea, although the identity of this metabolite needs confirmation. This suggested that ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl, the active ingredient in lauric arginate, may be rapidly metabolised by hydrolysis of the ethyl ester and lauroyl amide to arginine. The arginine, subsequently undergoes natural amino acid catabolism via the urea and citric acid cycles resulting in the formation of ^{14}C-carbon dioxide and ^{14}C-urea, which are excreted in the expired air and in urine respectively.

A possible route by which these terminal metabolites might be formed, is that following metabolism of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl to arginine, the arginine itself enters the urea cycle where it is degraded to ornithine and urea. The ornithine could then either be degraded further to citrulline and other amino acids in the urea cycle or to the metabolic intermediate α-ketoglutarate, which could then enter the citric acid cycle with subsequent metabolism to carbon dioxide[22,23].

Incorporation of the radiolabelled arginine into all the amino acids of the urea cycle would subsequently lead to a large number of endogenous products in the body. This would explain the high level of retention of radioactivity found in the carcass of the rats 5 days after dosing.

The high levels of radioactivity in liver were to be expected as this organ is the main site in the body for the enzymes of the urea cycle. The liver is also a major producer of proteins and bile and therefore radioactive amino acids formed as a result of lauric arginate metabolism and catabolism would be likely to accumulate here.

These results indicate that ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl, is rapidly converted to arginine and that it is likely to have toxicological properties similar to those of arginine.

In vivo and in vitro metabolism in the rat: evaluation of lauric arginate biotransformation pathways (Study LMA 033/012117, Huntingdon Life Science, Huntingdon, UK, 2001).

Introduction:

The metabolism of ^{14}C-LAE was investigated using *in vitro* and *in vivo* tests to investigate the biotransformation pathway of ^{14}C-LAE in rats and to assess if the primary metabolites of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl, the active ingredient in lauric arginate, were normal products of metabolism such as arginine and ornithine (Appendix 18).

Study Design:

^{14}C-LAE was administered orally at a nominal dose of 200 mg/kg bw to 6 male rats and the plasma analysed at various times after dosing. *In vitro* incubations of rat liver S-9 homogenate preparations and plasma with the test substance were also performed.

Good Laboratory Practice:

The study was conducted in compliance with the EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal management:

Male Sprague-Dawley (Crl:CD®(SD)BR) rats obtained from Charles River, Margate, Kent, UK were used. Animals were housed in pairs and allowed an acclimatisation period of 5 days prior to dosing. Room temperature was 19-23ºC, relative humidity 40 to 70% and a 12 hour artificial light/dark cycle was maintained. Six animals were treated, these aged 6-8 weeks and within the bodyweight range of 202-227 g on dosing.

[22] Schlede, E.; Mischnke, V.; Diener, W.; Kaiser, D. The International Validation study of the acute-toxic-class method (oral). *Arch. Toxicol.* 69, 659-670. 1994.
[23] Voet, D.; Voet, J.G. Amino acid metabolism in biochemistry. John Wiley & Sons, UK, 678-691. 1990.

Liver and plasma management:
In vitro (S-9 liver fraction): the liver of an untreated male rat was used to prepare the S-9 liver homogenate.

In vitro (control plasma): plasma from the untreated male Sprague-Dawley rat was collected by cardiac puncture and used immediately.

Test substance:
The test substance consisted of a mixture of non-radiolabelled LAE Batch 3036 [NB – purity 89.4%[24]], dissolved in a solution of radiolabelled ethyl-N^{α}-lauroyl-L-arginate HCl (Batch NPE/LMA001/65, purity >99%), in which the arginine portion of the molecule was uniformly labelled. The solution obtained was concentrated and the final volume adjusted. The test substance had a ^{14}C-LAE concentration of 40.4 mg/ml, a specific activity of 0.41 μCi/mg (0.015 MBq/mg) and had a radiochemical purity of 99.8%.

Test substance formulation:
In vitro (S-9 liver fraction): after concentration to near dryness [arginine-[U-^{14}C]] LAE stock solution was re-dissolved in methanol giving a final concentration of ^{14}C-LAE in solution of 0.98 mg/ml.

In vitro (control plasma): after concentration to near dryness [arginine-[U-^{14}C]] LAE was re-dissolved in sodium chloride (isotonic saline) giving a final concentration of ^{14}C-LAE in solution of 0.89 mg/ml.

In vivo: a formulation of the test substance was prepared on the day of dosing. After removal of the solvent, the test substance (^{14}C-LAE) was mixed with 1% w/v aqueous methyl cellulose (vehicle solution). The nominal concentration of this formulation was 80 mg ^{14}C-LAE /ml with a radiochemical purity of 99.7%.

Administration of the test substance and dose level:
In vitro (S-9 liver fraction): aliquots of S-9 liver fraction were treated with the test formulation to give a nominal treatment rate of 10 μg ^{14}C-LAE /ml.

In vitro (control plasma): control plasma was treated with ^{14}C-LAE to give a nominal treatment rate of 10 μg/ml.

In vivo: the test formulation was administered orally by gastric intubation to the rats at a dose volume of 2.7 ml/kg bw. The dose of ^{14}C- LAE received by each rat was 200 mg/kg bw.

Experimental details:
In vitro (S-9 liver fraction): samples of the S-9 liver homogenate treated with ^{14}C-LAE at a nominal 10 μg/ml were maintained in a shaking water bath at 37ºC. Samples were removed 4, 6 and 24 hours after treatment and immediately mixed with methanol. The solid debris was removed by centrifugation (twice) and the extracts were pooled prior to chromatographic analysis.

In vitro (control plasma): aliquots were taken for extraction immediately, 1 and 4 hours after incubation.

[24] The purity of this batch appears in the report as 93.2% because it is expressed as ethyl-N^{α}-lauroyl-L-arginate HCl·H_2O. When the purity is expressed as ethyl-N^{α}-lauroyl-L-arginate HCl it is 89.4%.

In vivo: blood samples were taken by cardiac puncture from pairs of rats 0.5, 1 and 4 hours after dose administration; blood samples were also taken from 4 untreated rats for control purposes. The plasma was separated for analysis.

Analytical methods employed to identify radioactive components:
The biotransformation of ^{14}C-LAE was assessed and the products of the metabolism of ^{14}C-LAE identified and quantified using Liquid Scintillation Counting (LSC); High Performance Liquid Chromatography (HPLC); Thin-Layer Chromatography (TLC) and Liquid Chromatography/Mass Spectrometry (LC/MS).

Results:

Rat S-9 liver *in vitro* experiments:

Identification of biotransformation products: In a control incubation (without S-9 mixture) no significant degradation of ^{14}C-LAE was observed. After an *in vitro* incubation of ^{14}C-LAE with rat S-9 liver fraction, eight radioactive compounds were detected. LC-MS, LC-MS/MS and HPLC and TLC co-chromatography confirmed that the 2 major compounds detected were unchanged ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and ornithine. Small portions of LAS and arginine ethyl ester were also identified in these S-9 extracts. The presence of urea, which was also confirmed as the main excretion product in urine from rats in a previous study (Appendix 17) was confirmed. A minor component, possibly N$^{\alpha}$-lauroyl-L-arginine-methyl ester was also identified, although this may be an artefact caused by the methylation of LAS during extraction with methanol and not a metabolite.

Quantification of the biotransformation products: TLC was used to quantify the biotransformation products after incubation of S-9 liver homogenate treated with 10 µg/ml ^{14}C-LAE. Four hours after treatment, 53.3% of ^{14}C-LAE was metabolised and at 24 hours only 25% was detected. The main metabolite identified was ornithine, which accounted for up to 29.3% of applied radioactivity (AR) 24 hours after treatment and a trace (<2% AR) of arginine. Urea increased with time from 3.8% at 4 hours to 7.8% of AR at 24 hours. LAS, arginine ethyl ester and 2 unknown compounds each accounted for less than 5% of AR at any time. Unextractable radioactivity was in the range of 9.2% to 14.4% of AR.

Rat plasma experiments *in vitro*:

Identification of biotransformation products: After treatment of rat plasma with ^{14}C-LAE at a nominal 10 µg/ml at least 5 components were detected:

1. unchanged ethyl-N$^{\alpha}$-lauroyl-L arginate HCl,
2. N$^{\alpha}$-lauroyl-L-arginine (LAS),
3. arginine and
4. a minor peak with the same retention time as ornithine.

Polar material was not identified but had a similar retention time to urea.
The plasma metabolite profiles were qualitatively similar to those observed in the *in vitro* S-9 liver experiments.

Quantification of the biotransformation products: The extraction of radioactivity was quantified at zero-time, 1 and 4 hours after treatment of rat plasma with a nominal 10 µg/ml ^{14}C-LAE.

At zero-time, the extraction of radioactivity was 99.5% and declined to 93.0% and 86.4% of AR by 1 and 4 hours after treatment, respectively.

The analysis of extracts showed that ^{14}C-ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl was rapidly hydrolysed to ^{14}C-LAS. The extraction of radioactivity as ^{14}C-ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and its metabolites quantified at various times after dosing is presented in the table below:

Table 7: *Percentage of applied radioactivity quantified at various times after dosing*

	% Applied Radioactivity				
Time	**Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl**	**LAS**	**Arginine**	**Ornithine**	**Polar material**
0 h	63.9	32.9	<0.2	<0.2	0.3
1 h	2.4	67.2	22.5	0.7	0.3
4 h	1.4	32.1	43.9	5.1	1.8

Non-extractable radioactivity increased from 3.8% at 0 hours to 15.4% 4 hours after dosing.

Rat plasma experiments *in vivo*:

Total radioactivity concentrations *in vivo*: In rats treated with a single oral dose of ^{14}C-LAE of 200 mg/kg bw, concentrations of total radioactivity in plasma increased from a mean of 14.2 μg equivalents ^{14}C-LAE/ml plasma 0.5 hours after dosing to a mean of 118 μg equivalents ^{14}C-LAE/ml at 4 hours after dosing.

Quantification of the biotransformation products: In plasma samples from rats that received a single oral dose, the extractability of total radioactivity (TRR) decreased markedly with time. At 0.5 hours after dosing a mean of 74.8% TRR was extracted while in contrast, 4 hours after dosing the mean TRR was 19.7%. Non-extractable radioactivity, increased from 21.6% TRR at 0.5 hours to 73.0% 4 hours after dosing.

Unchanged ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and LAS accounted for less than a mean of 10% TRR in all samples. Arginine was the major component present, accounting for a mean maximum of 48.4% TRR 0.5 hours after dosing which declined to 9.6% TRR at 4 hours. Ornithine accounted for a mean maximum of 7.7% TRR at 0.5 hours after dosing, declining to 1.5% TRR. Finally, the polar material increased to 17.4% TRR after 1 hour and then declined to a mean 7.4% TRR at 4 hours after dosing.

The % of various radioactive components in extracts of rat plasma at intervals after dosing are presented in Figure 6:

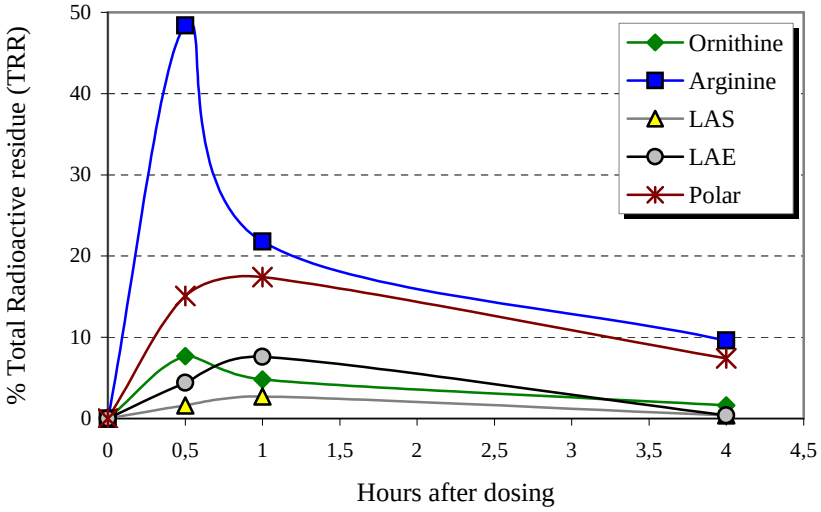


Figure 6: Percentage of radioactive components in extracts of plasma at intervals after a single oral dose of ^{14}C-LAE to rats

Conclusions:

This study showed that lauric arginate is rapidly metabolised by hydrolysis of the ethyl ester and lauroyl amide to arginine, which subsequently enters the natural urea cycle where it is hydrolysed to ornithine and urea. This, together with the previous report (Appendix 17) on the *in vivo* metabolism of lauric arginate in the rat, demonstrates that lauric arginate is metabolised primarily to naturally occurring amino acids. The amino acids in turn are incorporated into endogenous products and finally degraded to carbon dioxide through the normal mammalian pathways.

Lauric arginate: pharmacokinetics in rats *(Study LMA 057/053626, Huntingdon Life Sciences, Huntingdon, UK, 2005)***.**

Introduction:

The plasma pharmacokinetics of lauric arginate and its metabolite (N$^{\alpha}$-lauroyl-L-arginine, LAS) were investigated in rats after oral gavage administration of a range of dosages in various formulations (Appendix 23).

Study Design:

A preliminary study in male and female rats was performed to establish suitable dose levels and times for analysis of blood samples. A dose level of 40 mg/kg bw of lauric arginate was administered to groups of 4 male and female rats and the blood levels of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl (the active ingredient of lauric arginate) and LAS measured. Plasma levels of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl were generally below the limit of quantification but levels of LAS showed no difference between males and females, so only males were used for the main study. For the main study, groups of male rats received single oral (gavage) doses of 40, 120 and 320 mg/kg bw of lauric arginate in propylene glycol/water or 120 mg/kg bw in either glycerol/water or water alone. Blood samples were taken up to 8 hours post dose to assess the systemic exposure of male rats to ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and LAS.

Good Laboratory Practice:

The study was conducted in compliance with the EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal Management:
Male Sprague-Dawley (Crl:CD®(SD)BR) rats obtained from Charles River, Margate, Kent, UK were used. Animals were housed 4/cage and allowed an acclimatisation period of 5 days prior to dosing. Room temperature was 19-23ºC, relative humidity 40 to 70%, a 12 hour artificial light/dark cycle was maintained and there were approximately 15 air changes per hour. Animals, aged 7-10 weeks and within the bodyweight range of 213-228 g on dosing were used. Animals were monitored daily for behavioural change and on the day of dosing, were observed for clinical signs immediately after dosing and during blood sampling.

Test Substance:
LAE Batch 12547 (purity 91.87%).

Test substance formulation:
All doses in propylene glycol and glycerol were prepared at a ratio of 1 part lauric arginate to 3.6 parts solvent and then made up to volume with distilled water. The appropriate weight of lauric arginate and propylene glycol or glycerol was mixed until the lauric arginate had dissolved before water was then added. In water alone, lauric arginate was dissolved by sonication in 10 ml. All dose solutions were prepared on the day of dosing.

Administration of the test substance and dose level:
The dose solutions were administered orally using a graduated syringe with a rubber gavage tube. The dose volume was a nominal 5 ml/kg bw.

Sample collection:
Samples of blood were collected 30, 60, 90, 120, 240 minutes and 8 hours post dose.

Plasma was separated immediately after sampling and then processed.

Analytical methods employed:
Plasma concentrations of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and LAS were measured using a validated method (report LMA 055/042898, Huntingdon Life Science, Cambridgeshire, UK, 2004; Appendix 45).

Results:

Plasma concentrations of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl were generally close to or below the limit of quantification (<1 ng/ml) following administration of the propylene glycol/water formulation at all three dose levels. Following administration of 120 mg/kg bw in glycerol/water and water, plasma concentrations of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl were generally higher. Plasma concentrations of LAS were generally less variable.

Pharmacokinetics:
The mean C_{max} and AUC_8 values for the propylene glycol/water formulation are summarized below with standard deviations in parentheses:

Dose level (mg/kg bw)	Cmax (ng/ml)		AUC_8 (ng.h/ml)	
	Ethyl-N^{α}-lauroyl-L-arginate HCl	LAS	Ethyl-N^{α}-lauroyl-L-arginate HCl	LAS
40	2.02 (1.28)	24.2 (31.9)	- (-)	52.5 (45.0)
120	1.23 (0.29)	23.2 (2.5)	- (-)	103 (8)
320	2.60 (1.81)	96.9 (79.7)	7.50 (1.13)	315 (58)

- Could not be calculated.

Absorption was generally rapid, T_{max} was generally 0.5 or 1 hour post-dose for 40 and 120 mg/kg bw and in the range 0.5 to 4 hours for 320 mg/kg bw. An increase in the oral dose tended to prolong the absorption phase. T_{max} for LAS generally occurred at about the same time as for ethyl-N^{α}-lauroyl-L-arginate HCl.

Plasma concentrations of ethyl-N^{α}-lauroyl-L-arginate HCl were below the limit of quantification (<1 ng/mL) by 8 hours post-dose in all animals at all dose levels, indicating that the animals were only exposed to quantifiable concentrations of ethyl-N^{α}-lauroyl-L-arginate HCl for a relatively short time after dosing. However, plasma concentrations of LAS at 8 hours post-dose were quantifiable in all animals at the 120 and 320 mg/kg bw dose levels.

The relationships between mean C_{max}, AUC_8 and dose level are presented below:

Dose level (mg/kg bw)	Dose level ratio	Cmax ratio		AUC_8 Ratio	
		Ethyl-N^{α}-lauroyl-L-arginate HCl	LAS	Ethyl-N^{α}-lauroyl-L-arginate HCl	LAS
40	1.0	1.0	1.0	-	1.0
120	3.0	0.6	1.0	-	2.0
320	8.0	1.3	4.0	-	6.0

- Could not be calculated.

The rate of systemic exposure of male rats to ethyl-N^{α}-lauroyl-L-arginate HCl, characterised by Cmax, did not appear to increase consistently with increasing dose over the dose range 40 to 320 mg/kg bw of lauric arginate. The extent of systemic exposure of male rats to ethyl-N^{α}-lauroyl-L-arginate HCl, characterised by AUC_8, could not be estimated adequately for a number of animals, owing to the small number of quantifiable samples.

Consequently, it was not possible to make a formal assessment of dose-proportionality using this parameter. The mean rate (C_{max}) of systemic exposure of male rats to LAS was similar at 40 and 120 mg/kg bw but was higher at 320 mg/kg bw. When considered over the dose range 40 to 320 mg/kg bw this increase appeared to be less than the proportionate dose increment, however, there was marked inter-animal variation. The mean extent (AUC_8) of systemic exposure of male rats to LAS increased by slightly less than the proportionate dose increment over the dose range 40 to 320 mg/kg bw. Plasma concentrations of LAS were considerably higher than

those of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl, indicating that there was extensive conversion of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl to LAS. The terminal half-life could not be estimated adequately from the available data for either ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl or LAS.

The mean C_{max} and AUC_8 values after administration of lauric arginate at a dose of 120 mg/kg bw in propylene glycol/water, glycerol/water or in water are summarised below with standard deviations in parentheses:

Formulation	C_{max} (ng/ml)		AUC_8 (ng·h/ml)	
	Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl	LAS	Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl	LAS
Propylene glycol/water	1.23 (0.29)	23.2 (2.5)	- (-)	103 (8)
Glycerol/water	9.42 (3.54)	28.8 (4.6)	12.6 (4.5)	115 (18)
Water	10.6 (6.4)	31.2 (6.4)	8.78 (2.12)	109 (10)

- Could not be calculated.

T_{max} was generally 0.5 or 1 hour post-dose, indicating that absorption was generally rapid, and did not appear to be greatly influenced by the formulation. T_{max} for LAS generally occurred at about the same time as for ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl.

Plasma concentrations of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl were below the limit of quantification (<1 ng/ml) by 8 hours post-dose in all animals, indicating that the animals were only exposed to quantifiable concentrations of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl for a relatively short time after dosing. However, plasma concentrations of LAS at 8 hours post-dose were quantifiable in all animals and were generally similar for each formulation.

Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl, which is the ethyl ester of LAS, is very rapidly hydrolysed to LAS in the gastrointestinal tract and by tissue and plasma esterases. The low levels of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl detected are due to this rapid metabolism and do not give a good indication of the extent of absorption of lauric arginate. The levels of LAS, especially the AUC values, give a better indication of the absorption of lauric arginate and in general show that absorption is similar for all three formulations of lauric arginate studied here. It is known from studies in humans that LAS is in turn hydrolysed at the amide linkage to lauric acid and arginine, and that transient high blood arginine levels are observed. The present study gives no information about the absolute bioavailability of lauric arginate, but suggests that similar absorption is observed whether propylene glycol or glycerol are added to aid solubility in water.

Conclusions:

After administration of lauric arginate the rate and extent of systemic exposure of rats to ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl was low and there did not appear to be a consistent relationship of plasma ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl with dose following administration at dosages of 40, 120 and 320 mg/kg bw. This is related to the rapid hydrolysis of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl to LAS. For LAS however, the difference in the rate of exposure was much less marked; the C_{max} values (23.2, 28.8 and 31.2 ng/ml for the propylene glycol/water, glycerol/water and water formulations

respectively), and the AUC_8 values (103, 115 and 109 ng/ml for the propylene glycol/water, glycerol/water and water formulations respectively) were similar after all three formulations. Plasma levels of LAS, and in particular the AUC for LAS, give a better indication of the absorption of lauric arginate. The similarity in the values for the AUC of LAS shows that the absorption of lauric arginate is similar for all three of the formulations tested.

General conclusions of metabolism-toxicokinetic section:

The studies using a mixture of unlabelled lauric arginate and ^{14}C-ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl (arginine portion of the molecule uniformly radiolabelled) show that once ingested lauric arginate is well absorbed, rapidly metabolised, incorporated into naturally occurring products via the urea and citric acid cycles, distributed to liver, and slowly excreted as carbon dioxide via the expired air, and to a very low extent via the urine and faeces.

Based on the results obtained in the *in vitro* and *in vivo* studies (Appendix 17, 18) a pathway for lauric arginate metabolism has been proposed below and is presented in figure 7:

- *In vitro*: ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl is rapidly hydrolysed to arginine by loss of lauroyl side chain to form arginine ethyl ester and or cleavage of the ethyl ester to LAS. The arginine is then further hydrolysed to ornithine and urea.
- *In vivo*: ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl is rapidly metabolised to LAS and then to arginine and finally to ornithine. The radiolabelled ornithine would be incorporated into naturally occurring endogenous products via the urea and citric acid cycles.

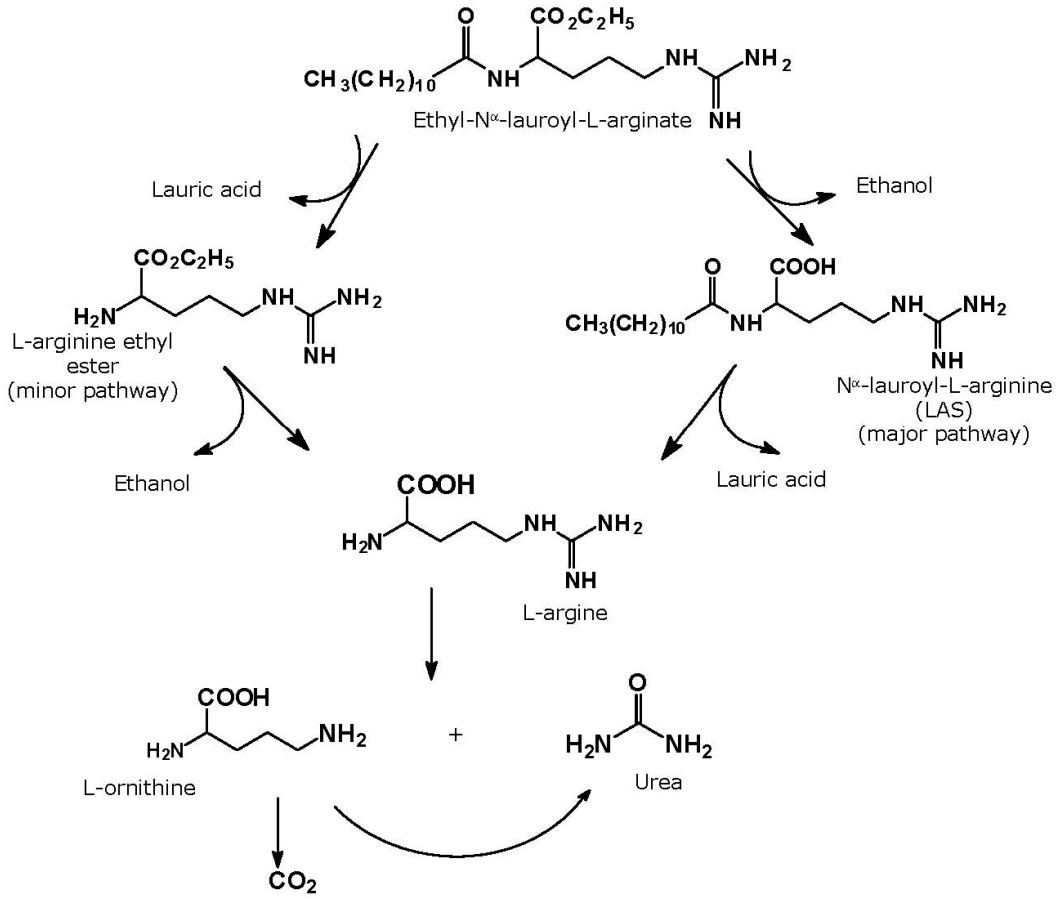


Figure 7: *Proposed biotransformation pathway for ethyl-N$^{\alpha}$-lauroyl-L-arginate in rats based on in vivo and in vitro studies* (Appendix 18, 21).

In the pharmacokinetic study of non-radiolabelled lauric arginate and LAS after a single oral dose of 40, 120 and 320 mg/kg bw of lauric arginate in propylene/glycol/water and 120 mg/kg bw in glycerol/water and water alone was administered to rats it was demonstrated that the metabolism of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl to LAS was rapid (Appendix 23). The rate and extent of exposure to ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl was low with all 3 formulations. This transient presence of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl in blood is due to its rapid metabolism in the GIT and by tissue and plasma esterases. Exposure to LAS was greater and C_{max} and AUC values were similar for the 3 formulations.

The similarity in the values for the AUC of LAS shows that the absorption of lauric arginate is similar for all three of the formulations tested.

TOXICITY

Acute Toxicity

As lauric arginate is intended for use in food and cosmetic products, the acute toxicity studies described in this section assessed 2 different exposure routes, oral and dermal, in rats. In addition, the acute oral toxicity of Mirenat-N, a formulation of lauric arginate, and N^{α}-lauroyl-L-arginine (LAS) the main metabolite of lauric arginate were also assessed in rats.

Oral Toxicity

Acute Oral toxicity of lauric arginate to the rat *(Study LMA 018/002881/AC, Huntingdon Life Science, Huntingdon, UK, 2000)***.**

Introduction:

This study assessed the acute oral toxicity of lauric arginate to a group of healthy rats, following the acute toxic class method (Appendix 46). This method is based on biometric evaluations and has been subjected to international validation[25]. The substance was administered by oral gavage to gain information on potential effects that may occur following accidental ingestion.

This study was conducted following:

- ECC methods for the determination of toxicity, Annex to Directive 96/54/EEC (Official Journal No. L248, 30.9.96), Part B. Method B.1 tris. Acute toxicity (oral) – acute toxic class method.
- OECD Guideline for Testing of Chemicals no. 423 "Acute Oral Toxicity – Acute Toxic Class Method" Adopted 22 March 1996.
- EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal Management:
Male and female CD rats of Sprague Dawley origin (Hsd:Sprague-Dawley (CD)) obtained from Harlan UK, Bicester, Oxon UK were used. An acclimatisation period of at least 5 days was allowed before starting any experimental work. Animals were housed 3/sex/cage with food and water available *ad libitum* except food overnight prior to dosing and for approximately 4 hours after dosing. Room temperature was 18.5-20ºC, relative humidity 34 to 55% and a 12 hour artificial light/dark cycle was maintained. Animals were 5-7 weeks of age and within the bodyweight range 90-118 g on selection before dosing.

Two groups, one of 3 male and a second of 3 female animals received 2000 mg/kg bw of lauric arginate in 1% w/v aqueous methylcellulose, by oral gavage at a dose volume of 10 ml/kg bw.

Test substance:
LAE Batch 2625 [purity 90.1%] was used as supplied.

Test substance formulation:
Lauric arginate was formulated at a concentration of 20% w/v in 1% w/v aqueous methylcellulose. The test substance formulation was prepared on the day of dosing.

[25] Schlede, E.; Mischnke, V.; Diener, W.; Kaiser, D. The International Validation study of the acute-toxic-class method (oral). *Arch. Toxicol.* 69, 659-670. 1994.

Stability and formulation analysis:
The stability of the test substance, the test substance in the selected vehicle and achieved concentration were not determined in this study.

Observations and terminal investigations:
Animals were observed soon after dosing and at frequent intervals for the remainder of day 1. Thereafter (days 2-14) animals were observed twice daily (morning and afternoon) and in the morning on day 15. Body weight was recorded on days 1, 8 and 15 and body weight changes calculated. At the end of the observation period (day 15), the animals were killed and submitted to a macroscopic *post mortem* examination in which the cranial, thoracic and abdominal cavities were opened and the organs examined.

Results:

Clinical signs and mortality:
There were no deaths among the 6 rats that received a single oral dose by gavage of 2000 mg/kg bw of lauric arginate. Therefore, it was not necessary to perform more than 2 steps in the procedure to evaluate the acute toxicity of lauric arginate.

The clinical signs of reaction to treatment observed in this study comprised piloerection and increased salivation, both evident in all rats, within five minutes of dosing. At this time, the gait of females only was waddling/unsteady and hunched posture was seen in males only. These signs persisted and were accompanied by hunched posture and soiled fur in females only later during day 1 (the soiled fur was associated with the increased salivation). All signs except piloerection (in all rats) and hunched posture (in females) had resolved by day 2 and these signs had resolved by day 3 (males) or day 4 (females).

Bodyweight:
All animals were considered to have achieved satisfactory bodyweight gains throughout the study.

Macroscopic examination:
No abnormalities were revealed in any of the animals at the macroscopic examination at study termination on day 15.

Conclusion:

The acute oral median lethal dose (LD_{50}) of lauric arginate to rats was demonstrated in this study to be greater than 2000 mg/kg bw. Thus, in accordance of the Directive 93/21/EEC, lauric arginate is regarded as not harmful if swallowed.

***Acute oral toxicity of Mirenat-N to the rat* (Study LMA 4/951314/AC, Huntingdon Life Science, Huntingdon, UK, 1995).**

Introduction:
This study assessed the toxicity of Mirenat-N, a formulation of lauric arginate consisting of a solution of lauric arginate in propylene glycol, following a single oral dose to rats. The substance was administered by oral gavage to gain information on potential effects that may occur following accidental ingestion (Appendix 47).

This study was carried out in accordance with:

- EEC Methods for the determination of toxicity, Annex Directive 92/69/ECC (OJ No. L383A, 29.12.92), Part B, Method B.1. Acute toxicity (oral), and
- EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal management:
Male and female CD rats of Sprague Dawley origin (Hsd/Ola:Sprague-Dawley (CD)) obtained from Harlan Olac Ltd., Bicester, Oxon, England were used. An acclimatisation period of 7 days was allowed before starting any experimental work. Animals were housed 5/sex/cage with food and water available *ad libitum* except overnight prior to dosing and for approximately 4 hours after dosing. Room temperature was set to achieve 19-25ºC, relative humidity 30 to 70%, 10-15 air changes /hour and a 12 hour artificial light/dark cycle was maintained. Animals were 4-7 weeks of age and within the bodyweight range 104-124 g on selection before dosing.

A group of 5 male and 5 female animals received a single dose of 2000 mg/kg bw of Mirenat-N, by oral gavage at a dose volume of 1.9 ml/kg bw.

Test substance:
The test substance was Mirenat-N Batch 00000003 [NB - 19.5% solution of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl in propylene glycol[26]].

Test substance formulation:
Mirenat-N, was used as supplied, and allowance was made for the specific gravity (1.0554) of the solution.

Stability and formulation analysis:
The stability and homogeneity of the test substance was not determined in this study. Formulation analysis was not relevant, the substance being used as supplied.

Observations and terminal investigations:
Rats were checked at least twice daily for mortality. Animals were observed soon after dosing and at frequent intervals for the remainder of day 1. On days 1-14, rats were observed once in the morning and again at the end of the working day, except on day 15 when rats were observed in the morning before dispatch to necropsy.

Bodyweight was recorded on days 1 (prior to dosing), 8 and 15. Individual bodyweight changes and were calculated. All rats were killed and subjected to a macroscopic examination, which consisted of opening the abdominal and thoracic cavities and examining the organs.

Results:

Clinical signs and mortality:
There were no deaths observed after the administration of the oral single dose of Mirenat-N at a dosage of 2000 mg/kg bw. Piloerection was observed within minutes of dosing in all rats and persisted throughout the remainder of Day 1. Recovery was complete by day 2. No other clinical signs were observed.

Bodyweight:
All rats achieved satisfactory bodyweight gains throughout the study.

[26] The purity of this batch appears in the report as 25% of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl by error due to the fact that HLS did not received the Certificate of Analysis.

Macroscopic examination:
On day 15, no macroscopic abnormalities were observed in any animal.

Conclusion:

The acute oral median lethal dose (LD_{50}) to rats of Mirenat-N was found to be greater than 2000 mg/kg bw. Thus, in accordance with definitions provided in the "Approved Criteria for Classifying Hazardous Substances" [NOHSC: 1008 (2004)], 3rd Edition for interpretation of Acute Toxicity, Mirenat-N is regarded as not having significant acute toxicity.

Summary of acute toxicity studies

The results of the acute toxicity studies are summarised below:

Table 8: *Summary of results*

Toxicity	Lauric arginate	Mirenat-N
Oral	**LD_{50} > 2000 mg/kg bw.** Piloerection until day 3 (males) and day 4 (females). Body weight gain satisfactory.	**LD_{50} > 2000 mg/kg bw.** Piloerection until day 1. Body weight gain satisfactorily.

Short-Term Toxicity

Subchronic Toxicity:

Subchronic toxicity studies in rats were performed to determine the toxicological profile of lauric arginate and Mirenat-N, a formulation of lauric arginate in propylene glycol, following repeated administration in the diet. Preliminary 4-week studies were performed using both test substances in order to determine suitable dosing levels for the subsequent subchronic 13-week studies.

Lauric arginate was administered at dietary concentrations of 25000, 37500 and 50000 ppm and 5000, 15000 and 50000 ppm for 4 and 13 weeks respectively. The dietary concentrations of Mirenat-N administered were 3200, 12800 and 50000 ppm in both studies.

Dose range finding/palatability study by dietary administration of lauric arginate to Han Wistar rats for 4 weeks *(Study LMA 030/000063, Huntingdon Life Science, Huntingdon, UK, 2000)***.**

Introduction:

The purpose of this study was to assess the palatability/systemic toxic potential of lauric arginate, on repeated dietary administration to Han Wistar rats for 4

consecutive weeks (Appendix 49). The dietary concentrations administered to rats were 25000, 37500 and 50000 ppm. The results of this study were used in the selection of appropriate concentrations for use in the subsequent 13-week dietary study of lauric arginate. As it was a preliminary study, methodology did not follow an official guideline. It was conducted in compliance with the principles of GLP though no specific study inspections were performed.

Experimental Procedure:

Animal Management:
Male and female Han Wistar rats obtained from Charles River, Margate, Kent, UK were used. Animals were housed 5/sex/cage with food and water available *ad libitum*. Animals were 42 days of age at the start of treatment and within the bodyweight range 100-138 g on selection 7 days before the start of treatment.

Three groups of 5 male and 5 female animals received lauric arginate by dietary admixture at concentrations of 25000, 37500 and 50000 ppm for 4 weeks. An additional group of 5 males and 5 females received untreated diet *ad libitum* and acted as a control group.

Test substance:
The test substance was LAE Batch 2625 (purity 90.1%).

Test substance formulation:
Lauric arginate as supplied was incorporated into the ground diet by initial preparation of a premix. Final concentrations were achieved by diluting aliquots of the premix with an appropriate amount of the basal diet and mixing. Diets were not analysed to determine stability, homogeneity or achieved dietary concentration.

Observations, laboratory and terminal investigations:
The animals were checked at least daily for clinical signs and mortality. Body weight was recorded once during acclimatisation, on Day 1 of treatment, at the end of week 1, twice weekly during Weeks 2-4, and immediately before necropsy. Food consumption was measured weekly and both efficiency of food utilisation and achieved dosage were calculated. Blood samples were taken from all animals after overnight fasting during week 4 to allow selected haematology and blood biochemistry parameters to be investigated. At the end of the treatment period, the animals were killed. Selected major organs were weighed. All animals were submitted to a full macroscopic *post mortem* examination. Selected tissue specimens were preserved and retained pending future requirements for microscopic examination.

Results:

Clinical signs and mortality:
There were no deaths during the treatment period. Brown staining of the muzzle was observed in most animals in all treated groups.

Bodyweight:
During days 1 to 7 of treatment:

- At 50000 ppm: animals lost or did not gain any weight.
- At 25000 or 37500 ppm: animals gained significantly less weight than the Controls.
- A dosage relationship was apparent.

From days 7 to 27:

- In treated males, the weight gain was similar to that of Controls.
- In treated females, the weight gain was higher than Control values.
- The weight gain for females given 37500 or 50000 ppm was markedly higher relative to the Control.

The overall gain (days 0-27):

- At 50000 ppm, weight gain of males was markedly reduced compared with the Controls.
- In treated females, weight gain was similar to or higher than that of the Controls.

Food consumption:
During the first week, the food consumption was markedly reduced in animals given 50000 ppm and reduced (concentration related) in animals given 37500 or 25000 ppm compared with the Controls. With the exception of females in week 2, treated animals continued eating less food than Controls. The overall intake was reduced for treated animals with males having showed a concentration-related depression in food intake while all females had similar low intakes. The food scatter was high during the first week for rats that received a dosage of 50000 ppm, for male rats given 25000 ppm and female rats given 37500 ppm. The effect persisted in males administered 25000 ppm of lauric arginate.

Food conversion efficiency:
In week 1, no determination of the conversion efficiency was possible for male rats given 50000 ppm. It was slightly reduced at 37500 ppm. In female rats, the conversion efficiency was markedly reduced at 50000 ppm during this week. Thereafter conversion efficiency in all treated groups was similar to or greater, particularly in animals given 50000 ppm, than in the control group.

Achieved Dietary Intake[27]:
Over the 4 weeks of treatment the group mean dosages, expressed as mg/kg bw/day of lauric arginate, were 2353, 3438 and 4273 for males and 2379, 3329 and 4641 for females respectively in groups 2-4. The ratios of the dietary concentrations were maintained.

Haematology:
After 4 weeks of treatment no significant findings clearly related to treatment were detected.

Blood chemistry:
After 4 weeks of treatment, when compared to controls:

- Total protein concentration was slightly low among treated males;
- Albumin and calcium concentrations were low in males receiving 37500 or 50000 ppm.
- In female rats, concentration-related slightly high alkaline phosphatase (50000 ppm), alanine amino-transferase and aspartate amino-transferase activities were evident in animals given 50000 ppm and in a few females given 37500 ppm.

Terminal observations:

[27] As calculated by HLS from the figures presented in the report.

Organ weights:

Analysis of organ weights after 4 weeks of treatment did not reveal any findings related to the treatment.

Macropathology:

There were no macroscopic findings that were considered to be related to treatment.

Conclusions:

When administered in the diet at a level of 50000 ppm for four-weeks, lauric arginate was palatable and well tolerated after the first week. Marked effects on bodyweight gain, food consumption and food conversion efficiency were evident during week 1 but recovery was seen thereafter. Clinical pathology changes were confined to slight effects possibly indicative of slight damage and/or functional changes in the liver. Based on these findings dietary concentrations of 5000, 15000 and 50000 ppm of lauric arginate were chosen for the subsequent 13-week study.

Toxicity study by dietary administration of lauric arginate to Han Wistar rats for 13 weeks *(Study LMA 031/004276, Huntingdon Life Science, Huntingdon, UK, 2001)***.**

Introduction:

The objective of this study (Appendix 10) was to assess the systemic toxic potential of lauric arginate in a 13-week dietary study in this strain of rat using concentrations of 5000, 15000 and 50000 ppm. The administration of lauric arginate in the diet at levels of up to 50000 ppm during 4 weeks had been previously shown to be palatable and tolerated by Han Wistar rats (Appendix 49).

This study was conducted in accordance with the requirements of the OECD Test Guideline No. 408 (revised 1998) and the Toxicological Principles for the Safety Assessment of Direct Food Additives and Colour Additives used in Food: Red Book 1 (1982).

The study was conducted in compliance with the EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal Management:
Male and female Han Wistar rats were obtained from Charles River, Margate, Kent, UK. An acclimatisation period of 13 days was allowed before starting any experimental work. Animals were housed 5/sex/cage with food and water available *ad libitum*. Room temperature was 19-25ºC, relative humidity 40 to 70%, there were approximately 15 air changes/hour and a 12 hour artificial light/dark cycle was maintained in the room. Animals were approximately 39-43 days of age and within the bodyweight range 127-173 g (males) and 114-151 g (females) at the start of treatment.

Three groups of 20 male and 20 female animals received the test substance by dietary admixture at the concentrations of 5000, 15000 and 50000 ppm for 13 weeks. An additional group of 20 males and 20 females received untreated diet *ad libitum* and acted as a control group.

Test substance:
The test substance was LAE Batch 2625 (purity 90.1%) and LAE Batch 3036 (purity 89.4%[28]).

Test substance formulation:
Lauric arginate as supplied was incorporated into the ground diet to provide the required concentrations by initial preparation of a premix. A conversion factor was applied for LAE Batch 3036 to take into account the water content of 4.1%. Final concentrations were achieved by diluting aliquots of the premix with an appropriate amount of basal diet and mixing. The suitability of the mixing procedure, the homogeneity and stability of the test substance in diet were determined pre-treatment, and the achieved concentrations were checked in weeks 1 and 12 of treatment. The admixed diets were stable for 23 days and achieved concentrations were satisfactory.

Observations, laboratory and terminal investigations:
The animals were checked at least twice daily for clinical signs and mortality and were also thoroughly examined weekly. Body weight was recorded once during acclimatisation, on day 1 of treatment, weekly throughout and immediately before necropsy. Food consumption was measured weekly and both efficiency of food utilisation and achieved dosage were calculated. Water consumption was monitored daily by visual inspection. Ophthalmology examinations were performed on all animals before treatment commenced and on 10/animals/sex from the control and high dietary-level groups in week 13. A Functional Observational Battery was included in this study. Hand and standard arena tests were performed once prior to treatment commencing and weekly thereafter and reflexes, responses and motor activity tests were performed once prior to treatment commencing and in week 12. Blood and urine samples were taken and collected from 10 animals/sex/group after overnight fasting and water deprivation during week 13 to allow selected haematology, blood biochemistry and urinalysis parameters to be investigated. At the end of the treatment period, the animals were killed. Selected major organs were weighed. All animals were submitted to a full macroscopic *post mortem* examination. Selected tissue specimens were preserved and submitted to a microscopic examination following appropriate processing.

Results:

Clinical signs and mortality:
During the treatment period there were no deaths. Other observations were:

- At 50000 ppm: the majority of male and female rats had ungroomed coats, associated with a high incidence of yellow staining of the coat on the ventral and dorsal body surfaces and perigenital area. Brown staining on the muzzle was also observed in animals of both sexes.
- At 15000 ppm: ungroomed coat was observed in two females while in males brown staining on the muzzle was observed.
- At 5000 ppm: there were no signs considered to be related to treatment.

Bodyweight:
During week 1, rats that received a dietary concentration of 50000 ppm had a marked loss of body weight (-16 and –13% of initial bodyweight of males and females respectively). In animals given 15000 ppm and in males receiving 5000 ppm, the body weight gain was significantly lower than that of the control animals. From week

[28] The purity of this batch appears in the report as 93.2% because it is expressed as ethyl-N^{α}-lauroyl-L-arginate $HCl \cdot H_2O$. When the purity is expressed as ethyl-N^{α}-lauroyl-L-arginate HCl it is 89.4%.

2, the body weight gains of animals given 50000, and males given 5000 ppm were similar to or higher (109 and 123% respectively) than those of the control group.

Overall, body weight of animals receiving 50000 and of males receiving 15000 ppm did not fully recover with total gains of 79 and 83% of Controls for males and females respectively receiving 50000 ppm, and 89% of Controls for males receiving 15000 ppm.

Food consumption:
During week 1, food consumption for animals that received 50000 ppm was markedly reduced (33% and 39%) for males and females respectively compared with Controls. Food consumption for animals receiving 15000 ppm and males receiving 5000 ppm was also slightly lower than that of the control group. These findings were associated with the amount of food scattered from the food hoppers, which was much higher for the treated animals than for the control groups. In animals given 50000 ppm food consumption remained low during the subsequent weeks but to a lesser degree (overall, 79 and 78% of controls for males and females respectively) and was probably not due to increased food scatter.

Food conversion efficiency:
During the first week of treatment this parameter could not be calculated for animals receiving 50000 ppm due to the loss in body weight. Thereafter, food conversion efficiency was slightly higher than, or similar to that of the Control groups.

Achieved Dosage:
The overall group mean dosages for the 13 weeks of treatment were 384, 1143, 3714 mg/kg bw/day of lauric arginate for males and 445, 1286 and 3915 mg/kg bw/day of lauric arginate for the equivalent female groups. The ratios of the dietary concentrations were maintained.

Functional Observational Battery:
There was no clear evidence of neurotoxicity in animals treated with lauric arginate for 13 weeks, at dietary levels of up to 50000 ppm.

Hand observations:
When compared with the control group, there were no differences other than those seen at the routine examinations (see clinical signs and mortality above).

Arena observations:
When 50000 ppm was administered, an increased incidence of slightly flattened gait (in males) or slightly elevated gait (in females) was observed in comparison with control groups during the 13 weeks of treatment. However, the incidence was inconsistent, tended to decrease over the last few weeks of study and the same gait abnormalities were observed in some control rats. As these slight, transient abnormalities related to body position during locomotion and not to a lack of co-ordination or to ataxia they were not considered to be of neurotoxicological significance. The changes may have been due to abdominal discomfort caused by irritation of the gastrointestinal tract.

Activity and rearing scores were markedly increased on several occasions during the first half of the treatment period for males and, to a lesser extent, for females, that received 50000 ppm. The differences were not detected in the more comprehensive assessment of motor activity performed in week 12. It is therefore possible that the increased activity in the arena was associated with the reduced body weight gain and decreased food consumption. Animals with reduced food intake may show higher than normal levels of exploratory activity when placed in a new environment.

Motor activity:
Motor activity scores were unaffected by treatment.

Ophthalmology:
There were no treatment-related ophthalmic findings.

Haematology:
Haematology investigations during week 13 of treatment revealed no significant treatment related findings. Slightly high mean cell haemoglobin, mean cell haemoglobin concentration, mean cell volume and slightly low total white blood cell and lymphocyte counts were observed in males receiving 50000 ppm. However the changes were marginal in degree and there were no changes evident in females and no effect of treatment was suspected.

Blood chemistry:
For animals receiving 50000 ppm and females that received 15000 ppm, low total protein concentration (50000 ppm) and slightly low albumin concentrations were observed. Slightly low cholesterol levels were also apparent in females receiving 50000 ppm. In the absence of effects on liver weight or associated histopathology these changes are not considered to be of toxicological significance.

Urinalysis:
The investigations in week 13, revealed a low pH in male rats only that received 15000 or 50000 ppm.

Organ weights:
There were no organ weight changes, which were clearly attributable to treatment.

Macroscopic pathology:
There were no treatment-related findings.

Histopathology:
The only changes were seen in the non-glandular region of the stomach, specifically in the area adjacent to the entry of the oesophagus. The predominant change was parakeratosis, which was present in the majority of male and female rats, that received 50000 ppm, and in a single female that received 15000 ppm. Ulceration was seen in a single male and two females, that received 50000 ppm and in a single male given 15000 ppm. In addition, erosions and epithelial hyperplasia were observed in female rats given the highest dietary level.

Conclusions:
When lauric arginate was administered in the diet at concentrations of up to 50000 ppm for 13 weeks it was generally well tolerated. Initial transient marked effects on body weight, body weight gain, food consumption and food conversion efficiency, associated with the palatability of the diet, were seen in animals given 15000 or 50000 ppm. From week 2 there was evidence of recovery. Evidence of mild toxicity was observed in-life for animals that received 15000 or 50000 ppm, with effects on appearance and clinical pathology parameters. Low protein and albumin concentrations were found, particularly in rats that received 50000 ppm. These results were indicative of an effect on liver metabolism or function. However in the absence of effects on liver weight and microscopic changes, these were considered of doubtful toxicological significance. The only histopathological findings considered to be related to treatment were restricted to the stomach of animals receiving 50000 ppm and comprised parakeratosis in most animals, ulceration in a few animals, erosions in a few females and epithelial hyperplasia in one female. One male and one female that received 15000 ppm of lauric arginate had ulceration and parakeratosis respectively.

The No-Observed-Adverse-Effect Level (NOAEL) identified in this study was 5000 ppm (384 and 445 mg/kg bw/day for males and females respectively).

Preliminary toxicity to rats by dietary administration of Mirenat-N for 4 weeks
*(Study LMA 2/952124, Huntingdon Life Science, Huntingdon, UK, 1995)***.**

Introduction:

Mirenat-N is a formulation of lauric arginate in a solution of propylene glycol. The purpose of this study was to assess the toxicity of Mirenat-N when administered continuously via the diet to groups of male and female rats for 4 weeks (Appendix 50).

Mirenat-N was administered in diet at concentrations of 3200, 12800 and 50000 ppm. The results obtained in this study were used in the choice of dietary concentrations for a subsequent toxicity study of 13 weeks.
As it was a preliminary study, an official test guideline or methodology was not followed.

The study was conducted in compliance with the EU, OECD and UK GLP Guidelines.

Animal Management:
Male and female Sprague-Dawley (Crl:CD®(SD)BR) rats obtained from Charles River, Margate, Kent, UK were used. An acclimatisation period of 13 days was allowed. Animals were housed 5/cage with food and water available *ad libitum*. Room temperature was 18-23ºC, relative humidity 46 to 72%, and a 12 hour artificial light/dark cycle was maintained. Animals were 42 days of age at the start of treatment and within the bodyweight range 173-207 and 137-163 g for males and females respectively on Day 1 of treatment.

Three groups of 5 male and 5 female animals received the test substance by dietary admixture at the concentrations of 3200, 12800 and 50000 ppm for 4 weeks. An additional group of 5 males and 5 females received untreated diet *ad libitum* and acted as a control group**.**

Test substance:
The test substance was Mirenat-N Batch 0000003 [a 19.5% solution of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl in propylene glycol[29]].

Preparation of test substance formulation:
A pre-mix was prepared weekly by adding the test substance as supplied, directly to the maintenance diet and mixing. The required concentrations were then prepared by direct dilution of the pre-mix with further quantities of untreated diet. Diets were not analysed to determine stability, homogeneity or achieved dietary concentration.

Observations, laboratory and terminal investigations:
The animals were checked at least daily for clinical signs and mortality. Body weight was recorded on:

1. Allocation to groups,
2. On Day 1 of treatment and then
3. Weekly throughout the study period as well as

[29] The purity of this batch is erroneously stated in the report as 25%. The error was caused by HLS not receiving the Certificate of Analysis.

4. Immediately before necropsy.

Food consumption was measured weekly and both efficiency of food utilisation and achieved dosage were calculated. Blood samples were taken from all animals during week 4 to allow selected haematology and blood biochemistry parameters to be investigated. At the end of the treatment period, the animals were killed. Selected major organs were weighed. All animals were submitted to a full macroscopic *post mortem* examination. Selected tissue specimens were preserved and retained pending the need for future microscopic examination.

Results:

Clinical signs and mortality:
No mortality or treatment-related clinical signs were observed during the 4 weeks of treatment.

Bodyweights, food consumption and efficiency of food utilisation:
There were no treatment-related effects at any dietary level.

Haematology and biochemistry parameters:
There were no parameters that were considered to have been affected by treatment.

Organ weights:
No treatment-related differences in the weight of any tissue were observed.

Macroscopic pathology:
There were no macroscopic changes considered to be attributable to treatment.

Conclusions:

There were no treatment-related findings at any dietary level and thus the No-Observed Effect Level (NOEL) in this 4 week study was 50000 ppm of Mirenat-N, equivalent to an average intake of 5269 and 5846 mg/kg bw/day for males and females, respectively.

Since 50000 ppm of Mirenat-N is the maximum dietary inclusion level, which can be used without compromising the nutritional quality of the diet, a high dietary level of 50000 ppm was selected for the 13 week study in the rat.

Toxicity to rats by dietary administration of Mirenat-N for 13 weeks *(Study LMA 3/961342, Huntington Life Science, Huntingdon, UK, 1996)***.**

Introduction:

Mirenat-N is a formulation of lauric arginate in a solution of propylene glycol. The objective of this study was to assess the toxicity of Mirenat-N (Appendix 51) when administered continuously in the diet for 13 weeks. The dietary levels, 3200, 12800 and 50000 ppm, were selected on the basis of the results of the preliminary study (Appendix 50).

This study was conducted in accordance of with the following guidelines:

- EEC guidelines as contained in the notes for guidance concerning the application of the Annex of Directive 87/302/EEC.

- OECD Guideline for Testing of Chemicals, No. 408, "Subchronic Oral Toxicity – Rodent", dated 12 May 1981.
- EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal Management:
Male and female Sprague-Dawley (Crl:CD®(SD)BR) rats obtained from Charles River, Margate, Kent, UK were used. An acclimatisation period of 21 days was allowed. Animals were housed 5/sex/cage with food and water available *ad libitum*. Room temperature was 19-23ºC, relative humidity generally 45 to 65%, and a 12 hour artificial light/dark cycle was maintained. Animals were 49 days of age at the start of treatment and within the bodyweight range 209-278 (males) and 151-199 (females) g on Day 1 of treatment.

Three groups of 10 male and 10 female animals received the test substance by dietary admixture at the concentrations of 3200, 12800 and 50000 ppm for 13 weeks. An additional group of 10 males and 10 females received untreated diet *ad libitum* and acted as a control group.

Test substance:
The test substance was Mirenat-N Batch 0000001 [a 19.4% solution of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl in propylene glycol[30]].

Preparation of test substance formulation:
Two pre-mixes were prepared weekly by adding diet to the test substance, thoroughly stirring by hand and mixing. The second premix was made by diluting the first with more diet and mixing. Prior to starting the study, samples of the formulations were checked by chemical analysis in order to confirm that the formulation method was acceptable and that the homogeneity and stability of the formulations were satisfactory. The accuracy of the preparation was also checked in weeks 1 and 10 and found to be satisfactory.

Observations, laboratory and terminal investigations:
The animals were checked at least once daily for clinical signs and mortality (twice) and were also palpated daily (Monday–Friday) for 4 weeks and thereafter weekly. Body weight was recorded on allocation to groups, on day 1 of treatment, weekly throughout and immediately before necropsy. Food consumption was measured weekly and both efficiency of food utilisation and achieved dosage were calculated. Water consumption was monitored daily by visual inspection and accurately during week 12. Ophthalmology examinations were performed on all animals before treatment commenced and on all animals from the control and high concentration-level groups in week 13. Blood and urine samples were taken and collected respectively from all animals after overnight fasting and water deprivation (urinalysis) during week 13 to allow selected haematology, blood biochemistry and urinalysis parameters to be investigated. At the end of the treatment period, the animals were killed. Selected major organs were weighed. All animals were submitted to a full macroscopic *post mortem* examination. Selected tissue specimens were preserved and submitted to a microscopic examination following appropriate processing.

Results:

[30] The purity of this batch is erroneously stated in the report as 25%. The error was caused by HLS not receiving the Certificate of Analysis.

Mortality:
There was one death amongst control males during week 1. This death was clearly not related to treatment with the test substance. Post mortem examinations revealed rupture of the liver as the cause of death.

Clinical signs:
There were no treatment-related clinical signs noted during the study. A slightly higher incidence of hair loss amongst females receiving 12800 and 50000 ppm of Mirenat-N was considered coincidental because there was no obvious pattern or increase in severity with increasing dietary level.

Bodyweights:
The overall mean bodyweight gain of males was similar to concurrent controls. The overall mean body weight gain for all treated groups of females was slightly lower than the concurrent controls. However, there was no relationship to dietary level. In the absence of this relationship and any effect in the treated males, the lower body weight gain in females is of uncertain relationship to treatment.

Food consumption:
The mean cumulative food intake of animals in treated groups was unaffected by treatment.

Food conversion efficiency:
The efficiency of food utilisation for all treated females was marginally inferior to that of the concurrent controls. However, there was no apparent relationship to dietary level. The efficiency of food utilisation of treated males was comparable to that of the concurrent controls.

Achieved intakes:
The achieved intakes of Mirenat-N followed the expected pattern for a fixed level dietary study, with exposure to the test substance being higher during the period of fastest growth. The intended interval (approximately 4 fold) in achieved intakes between the groups was well maintained throughout the study with overall mean intakes of 220, 904 and 3324 mg/kg bw/day for males and 262, 1067 and 3927 mg/kg bw/day for females in the treated groups.

Water consumption:
Group mean water intake by males given 50000 ppm was slightly higher than that of Controls. In all other groups the mean water intake was comparable with concurrent controls in all groups.

Ophthalmoscopy:
Examination of the eyes of control group animals and those receiving 50000 ppm during week 13 of treatment did not reveal any lesions considered to be attributable to treatment.

Haematology:
When compared with control animals, slightly lower total white blood cell counts were noted amongst males and females receiving 12800 and 50000 ppm of Mirenat-N. However, there was no consistency in the cell type contributing to the lower total cell count and as such these effects were of uncertain toxicological importance.

Biochemistry:
The differences in some parameters observed in groups receiving 12800 and 50000 ppm when compared with the control group, were slight and inconsistent across the sexes and hence no relationship to treatment is suspected.

Urinalysis:
The only evidence of an effect of treatment was a slight increase in some individual and group mean urine volumes, which was associated with the slightly higher water intake of males given 50000 ppm when compared with controls. No significant differences in urine volume or other parameters were seen in the other treatment groups compared with controls.

Organ weights:
There was a slightly higher mean adjusted (bodyweight as covariate) liver weight amongst females receiving 50000 ppm of Mirenat-N when compared to concurrent controls. No similar increase was apparent in the males at this dietary level and, since there were no microscopic changes detected in the liver and individual liver weights were within the same range as the controls, this finding is of uncertain toxicological significance.

Macroscopic pathology:
The only change observed was an increased incidence of alopecia amongst female rats receiving 12800 or 50000 ppm of Mirenat-N. In isolation, this change is considered to be of uncertain biological significance.

Microscopic pathology:
No treatment-related changes were detected in any of the tissues examined.

Conclusions:

With the possible exception of the equivocal lower body weight gain in females given Mirenat-N at any dietary level and the slightly higher water consumption and urine volume of males given 50000 ppm there were no conclusive signs of toxicity amongst treated animals when compared with the concurrent Controls.

The No-Observed-Adverse-Effect Level (NOAEL) for continuous administration of Mirenat-N to rats for 13 weeks is considered to be 12800 ppm (904 and 1067 mg/kg bw/day for males and females respectively) based on an increased incidence of alopecia.

Summary of the results of the subchronic toxicity studies:

The results of the subchronic toxicity studies are summarised below:

Table 9: *Summary of results obtained in the subchronic toxicity studies*

Treatment Period	**Lauric arginate**	**Mirenat-N**

4 weeks	Dietary concentrations: 25000, 37500 and 50000 ppm were well tolerated.	Dietary concentrations: 3200, 12800 and 50000 ppm were well tolerated.
13 weeks	Dietary concentrations of 5000, 15000 and 50000 ppm. **NOAEL:** 5000 ppm (384 and 445 mg/kg bw/day for male and female rats respectively).	Dietary concentrations of 3200, 12800 and 50000 ppm. **NOAEL:** 12800 ppm (904 and 1067 mg/kg bw/day for male and female rats respectively) based on increased incidence of alopecia.

Long-Term Toxicity

Chronic toxicity *(Study LMA 050/042556, Huntingdon Life Science, Huntingdon, UK, 2000)***.**

Introduction:

The objective of this study was to assess the systemic toxic potential of lauric arginate when administered in the diet to rats for 52 weeks (Appendix 11). The concentrations of lauric arginate in the diet used in this study were 2000, 6000 and 18000 ppm.

This study was conducted in accordance with the requirements of OECD Test Guideline No. 452 (adopted 1981) and EC B30 Annex V to Directive 67/548/EEC as amended in Directive 88/302/EEC and in compliance with EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal Management:
Rats of the Crl:CD®(SD)IGS BR strain obtained from Charles River (UK) Ltd., Margate, Kent, England were used and were allowed to acclimatise for 15 days before treatment commenced. Animals were housed four of one sex per cage with free access to food, except overnight before routine blood sampling and urine collection, and water.

The temperature and relative humidity controls were set within the range of 19 to 23ºC and 40 to 70% respectively. Artificial lighting was controlled to give a cycle of 12 hours light/dark. At the start of treatment the rats were aged 50 to 57 days and the mean bodyweights for males and for females were 273 g and 200 g respectively.

Three groups of 20 male and 20 female rats received lauric arginate in the diet for at least 52 consecutive weeks at concentrations of 2000, 6000 and 18000 ppm. An additional group of 20 male and 20 female animals received untreated diet and constituted the Control group.

Test substance:
The test substance was LAE Batch 7446 (purity 88.2%).

Test substance formulation:
The test substance was used as supplied. All formulated diets were prepared freshly every two weeks. The homogeneity and stability of the lowest (2000 ppm) dietary level was confirmed prior to the start of the treatment. The highest levels used in this

study had been confirmed as part of the previous 13-week study (Appendix 10). Samples of each formulation prepared for administration in weeks 1, 13, 26, 39 and 51 of treatment were analysed for achieved concentration of the test substance. The admixed diets were stable for 22 days and achieved concentrations were satisfactory.

Observations, laboratory and terminal investigations:
Animals were inspected visually at least twice daily for mortality and evidence of clinical signs (ill-health or reaction to treatment). Debilitated animals were observed carefully and, where necessary, isolated to prevent cannibalism. Animals judged *in extremis* were killed. Before treatment commenced and weekly thereafter a detailed physical examination and arena observations for neurobehavioural screening were performed on 10 animals/sex/group. In week 49 sensory activity, forelimb and hindlimb grip strength and motor activity were measured in the same 10 animals/sex/group.

Rats were weighed one week before treatment commenced, on the day treatment commenced, weekly for the first 26 weeks, once every 4 weeks thereafter and before necropsy.

Food consumption was measured for the week before treatment started, weekly for the first 26 weeks and once every 4 weeks thereafter. The mean weekly consumption per animal was calculated, together with group mean weekly food conversion efficiency and group mean weekly achieved dosage.

Ophthalmic examination was performed on all animals before treatment commenced and on all animals in the control and 18000 ppm groups during week 51.

Blood and urine samples were taken/collected from 10 animals/group after overnight fasting during weeks 14, 26 and 52 and 12, 25, 51 respectively of treatment and selected haematology (peripheral blood), blood chemistry and urinalysis parameters investigated.

During week 52 of treatment, blood samples were obtained from 3 male and 3 female animals in each treated group at 18:00, 22:00, 02:00, 06:00, 10:00 and 14:00 for bioanalytical and toxicokinetic investigations. Samples from all treated groups were analysed. Samples from untreated control groups were retained for possible method calibration use.

After 52 weeks of treatment all animals were subjected to a detailed necropsy. Samples of bone marrow were obtained from the tibia bone during necropsy of all animals killed. A full macroscopic examination of the tissues was performed. Selected major organs were weighed (paired organs together) and selected tissue specimens, including abnormal tissues, were preserved and submitted to microscopic examination following appropriate processing.

Results:

Toxicokinetics:
Blood samples were taken during week 52 in order to assess the systemic exposure of male and female rats to ethyl-N^{α}-lauroyl-L-arginate HCl, the active ingredient of lauric arginate, and its metabolite N^{α}-lauroyl-L-arginine (LAS). Plasma concentrations of ethyl-N^{α}-lauroyl-L-arginate HCl and LAS were measured using a validated method (report LMA 055/042898, Huntingdon Life Science, Cambridgeshire, UK, 2004; Appendix 45).

During week 52 inter-individual variation in plasma ethyl-N^{α}-lauroyl-L-arginate HCl concentrations was high but the inter-individual variation in plasma LAS

concentrations was lower. At the lowest dietary concentration, 2000 ppm, plasma concentrations of ethyl-N^{α}-lauroyl-L-arginate HCl and LAS were below limits of quantification (i.e.: <1 ng/ml for ethyl-N^{α}-lauroyl-L-arginate HCl and <5 ng/ml for LAS). The statistical assessment of the data was therefore restricted to groups that received dietary concentrations of 6000 and 18000 ppm.

The maximum and minimum mean plasma concentrations of ethyl-N^{α}-lauroyl-L-arginate HCl and LAS are reported in the following table:

Table 10*: Plasma concentrations of ethyl-N^{α}-lauroyl-L-arginate HCl and LAS*

Dietary concentration (ppm)	***Ethyl-N^{α}-lauroyl-L-arginate HCl***				**LAS**			
	C_{max} (ng/ml)		**C_{min} (ng/ml)**		**C_{max} (ng/ml)**		**C_{min} (ng/ml)**	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
2000	1.15	11.3	--	--	--	12.7	--	--
6000	6.92	22.9	--	--	18.7	26.4	--	9.30
18000	17.6	26.3	--	--	62.2	59.6	19.2	32.2

-- Mean not calculated as 50% or more values were below the level of quantification (BLQ).

During Week 52 the relationships between the maximum mean plasma concentration of ethyl-N^{α}-lauroyl-L-arginate HCl, LAS and achieved dietary intake were as follows:

Table 11*: Relationship between maximum mean plasma concentrations*

Dietary concentration (ppm)	Achieved intake ratio		**C_{max} ratio**			
			Ethyl-N^{α}-lauroyl-L-arginate HCl		**LAS**	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
6000	1	1	1	1	1	1
18000	3.0	3.0	2.5	1.1	3.3	2.3

The rate of systemic exposure of rats (C_{max}) to ethyl-N^{α}-lauroyl-L-arginate HCl and LAS generally appeared to increase approximately proportionately with increasing dose over the dietary concentration range 6000 to 18000 ppm, except for females where the increase was less than the proportionate dose increment. Overall, there was no statistically significant ($p>0.90$) evidence of non-proportionality, which in the case of lauric arginate may have been due, at least in part, to the high inter-animal variability observed.

The areas under the mean plasma ethyl-N^{α}-lauroyl-L-arginate HCl and LAS concentration-time curves estimated over a 24-hour (AUC_{24}) interval during Week 52 are summarised in the following table:

Table 12*: AUC_{24} of ethyl-N^{α}-lauroyl-L-arginate HCl and LAS*

Dietary concentration (ppm)	AUC_{24} (ng·h/ml)			
	Ethyl-N^{α}-lauroyl-L-arginate HCl		**LAS**	
	Males	**Females**	**Males**	**Females**
2000	19.5*	78.3*	46.8*	169
6000	66.8	130	286	368
18000	190	244	960	1130

** This value should be interpreted with caution as it represents the minimum estimate of the systemic exposure due to the high proportion of concentrations that were below the limit of quantification.*

The relationships between the areas under the mean plasma concentration-time curves (AUC_{24}) for ethyl-N^{α}-lauroyl-L-arginate HCl and LAS and the dietary concentrations of 6000 and 18000 ppm during Week 52 were as follows:

Table 13*: Relationship between AUC_{24} values*

Dietary Concentration (ppm)	Achieved intake ratio		AUC_{24} ratio			
			Ethyl-N^{α}-lauroyl-L-arginate HCl		**LAS**	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
6000	1	1	1	1	1	1
18000	3.0	3.0	2.8	1.9	3.4	3.1

The extent of systemic exposure of rats to ethyl-N^{α}-lauroyl-L-arginate HCl and LAS (AUC_{24}) appeared to increase approximately proportionately with increasing dose over the dietary concentration range 6000 to 18000 ppm, except the AUC_{24} values for females where the increases appeared to be less than the proportionate dose increment. In general, there was no statistically significant evidence of non-proportionality ($p>0.52$), which in the case of lauric arginate may have been due, at least in part, to the high inter-animal variability observed.

The dose-adjusted rate (C_{max}) and extent (AUC_{24}) of systemic exposure for female rats to ethyl-N^{α}-lauroyl-L-arginate HCl and LAS were generally similar to those indices of exposure in male rats with the exception of the C_{max} values for ethyl-N^{α}-lauroyl-L-arginate HCl, and overall there was no statistically significant evidence for any sex-related differences in systemic exposure ($p>0.28$).

Mortality:

There were six deaths during the study, none of which were considered to be attributable to treatment.

Clinical signs:

18000 ppm: during Weeks 1 to 13, females showed consistently higher weekly incidences of brown fur staining than control. In addition, during Weeks 4 to 12, females also showed higher weekly incidences of ungroomed coat than controls. The number of animals showing these signs in any given week fluctuated and there was no clear evidence of progression over time from Week 14.

6000 ppm: in Weeks 1 to 13, females showed weekly incidences of brown fur staining that were higher than controls but the weekly incidences were generally lower than at the highest dietary level and the staining was mainly confined to the head region. Thereafter, the weekly incidences were generally similar to those of controls.

2000 ppm: both sexes did not show any clear consistent differences from controls with regard to brown fur staining or ungroomed appearance.
There were no other differences from controls in the incidence and/or type of clinical signs, which were considered to be related to treatment.

Body weights:

18000 ppm: in the first week of treatment, males showed notably lower group mean gain than the control group. Thereafter, there was good improvement in body weight although the overall gains were still lower than control for Weeks 1-26 and 26-52 but this was due to lower food intake than controls.

Females also showed an initial lower bodyweight gain compared to controls for the first 3 weeks (Weeks 0 (day 1) to Week 3) of treatment. From Week 3 gains improved and group mean gain in Weeks 3-26 was similar to controls. Significantly lower group mean gains were again noted in Weeks 26-52; the magnitude was similar to the difference from control recorded in Weeks 0-3.

6000 ppm: similar effects on body weight gain to those reported at 18000 ppm were seen for both sexes, although the magnitude of the initial differences from controls were less than those reported at 18000 ppm.

2000 ppm: the weight gain of both sexes was unaffected by the treatment.

Food consumption:

18000 ppm: during the first week both sexes ate notably less food in comparison with pre-dose values and the concurrent controls. From Week 2 of treatment there was an appreciable improvement in food intake and it was considered that after the first week there was no toxicologically significant effect on the food consumption.

6000 ppm: although food intake for males was slightly lower than concurrent controls throughout the reduction in food intake was considered not to be of toxicological importance. There was considered to be no effect of treatment on the food intake of females as, although the mean food intake was lower than controls throughout, this difference was also seen during the pre-dose period.

2000 ppm: no effect of treatment in either sex was observed.

Food conversion efficiency:

18000 and 6000 ppm: in Week 1 food conversion efficiency for both sexes was lower than control but from Week 2 to 26 it was similar to controls.

2000 ppm: no effects of treatment for either sex were observed.

Achieved dosage:

Overall group mean achieved intakes at 2000, 6000 and 18000 ppm for Weeks 1 to 52 were 106, 307 and 907 mg/kg bodyweight/day for males and 131, 393 and 1128 mg/kg bodyweight/day for females.

Neurobehavioural screening:

No effect of treatment was observed in the detailed physical examination, in arena observations, in sensory reactivity and in grip strength.

The motor activity of females were unaffected but males at 18000 ppm showed higher total high and low beam scores compared with control at 1 hour and from 12 minutes respectively. Males at 2000 or 6000 ppm also showed higher total high and low beam scores compared with controls but in these cases, the differences from control were attributed to high values for a few individual animals and were not considered to be attributable to treatment.

Ophthalmic examination:

After 52 weeks of treatment there were no treatment related ocular changes.

Haematology-bone marrow:

There were no clear effects of treatment on the bone marrow.

Haematology peripheral blood:

There were no effects considered to be of toxicological significance. There were treatment–related effects on white cell parameters for both sexes. In week 26, males and females at 18000 ppm showed lower total white blood cell counts than control but this was only present in males in week 52. The differences were mainly due to lower neutrophils or lymphocytes and occasional effects on monocytes or large unstained cells though no consistent effects were observed. However, in the absence of any concentration related trends or treatment related effects on bone marrow and with the absence of histopathology associated with lymphoid tissue, the white cell disturbances were not considered to be of toxicological importance.

There were no effects of treatment on the red blood cell or clotting parameters throughout the study.

Blood chemistry:

Higher group mean urea values were noted for all groups of treated females in Week 52, a dietary level relationship was evident between 6000 and 18000 ppm, but not between 2000 and 6000 ppm. The mean value for females at 18000 ppm achieved statistical significance in comparison with controls. There were no effects of treatment on the other parameters examined.

Urinalysis:

The composition of the urine was considered to be unaffected by treatment.

There were no treatment related effects on the quantitative and semi-qualitative parameters.

Organ weights:

There were no clear effects of treatment on organ weights. A lower than control mean absolute adrenal weight was noted for females receiving 18000 ppm. The difference in adrenal weights was not considered to be attributable to treatment as there were 2 high values among the controls and 2 low values in the 18000 ppm group.

Macroscopic pathology:

Depressions on the epithelial aspect of the forestomach (in the oesophageal groove) were observed in 12/19 male and 9/19 female rats at 18000 ppm; in 5/20 male and 6/20 female rats at 6000 ppm and in 1/18 male and 5/19 female rats at 2000 ppm compared with 0/19 male control rats and 2/20 female control rats.

A dietary concentration related higher incidence of thickening of the uterine cervix was noted for all treated female groups compared with the control. The incidence and distribution of all other findings were considered to fall within the expected background range of macroscopic changes.

Histopathology:
Treatment related findings:

- Stomachs:
  Lesions were observed in the non-glandular epithelium of the stomach in several animals treated with lauric arginate. Erosion was seen in 1/19 females at 18000 ppm. Ulceration was present in 3/19, 3/20, and 6/19 female animals at 2000, 6000 and 18000 ppm, in 1/20 female controls, and in 3/20 males at 6000 ppm. Re-epithelialisation was seen in 1/20 and 3/19 males treated with 6000 ppm and 18000 ppm respectively and 3/19, 2/20 and 5/19 females from the 2000, 6000 and 18000 ppm groups respectively and in 1/20 female controls. Epithelial hyperplasia was reported in 1/18, 3/20 and 9/19 male animals from the 2000, 6000 and 18000 ppm groups respectively, 3/19, 5/20 and 8/19 female animals in the 2000, 6000 and 18000 ppm groups respectively and in 1/20 female controls.

  When compared with the control groups the differences in the number of affected animals was only statistically significantly greater for animals receiving 18000 ppm. These changes were accompanied by subepithelial/submucosal inflammation and subepithelial fibrosis of the non-glandular epithelium, along with some inflammation of the underlying muscle and serosal layer. These lesions in association with the accompanying subepithelial/submucosal inflammation, subepithelial fibrosis and inflammation of the muscle and serosal layers of the same area, broadly reflect the depressions noted in the forestomach recorded at necropsy.

  There were no changes within the glandular region of the stomach that were considered to be treatment-related.

- Other findings:
  Four cases of mammary adenocarcinoma were reported in females, one at 2000 ppm, one at 6000 ppm and two at 18000 ppm. The relatively low incidence of these tumours, and the absence of any other treatment related changes in the mammary tissues, does not suggest any relation to the administration of lauric arginate. Historical control data show a spontaneous incidence of these tumours with 2 being observed in 179 control females.

Discussion:

The exposure of rats to lauric arginate at the different dose levels tested for up to 52 weeks was generally well tolerated. However, toxicologically significant effects were observed at the highest dietary level.

During the initial stages, dosage-related lower bodyweight gains were seen for both sexes receiving 6000 or 18000 ppm, with animals treated at 18000 ppm showing gains which were less than 50% of the concurrent controls. In Week 1, decreased food intake was also clearly apparent for both sexes at 18000 ppm but the Week 1 food efficiency data indicated that the initial lower gains were not solely due to lower food intake. After Week 1, the efficiency of food utilisation for both sexes at 6000 or 18000 ppm was generally similar to controls, indicating a balance between lower food intake and reduced bodyweight gain. It is possible that the lower food intake noted after Week 1 was at least partly due to a mild unpalatability of the diet.

The only other toxicologically significant effect observed was in the forestomach in animals of the 18000 ppm group. The lesions were consistent with local irritation of the mucosa with hyperplasia, erosions and ulcerations, with evidence of healing and re-epithelialisation. The incidence of these lesions was only statistically significantly different from controls at this dietary level. The changes seen in the stomach were confined to a specific area of the forestomach, the oesophageal groove and are thought to represent a local response to irritation. Thus, they are considered not to be indicative of systemic toxicity. There was no correlation between the individual animals, which showed lower bodyweight gain, poor grooming and/or brown fur staining, and the presence of these stomach lesions. Nor was there any correlation between the animals, which showed stomach lesions and those, which exhibited white blood cell and/or biochemical disturbances. This further supports the hypothesis that the stomach lesions are not indicative of systemic toxicity.

Conclusion:

Treatment related effects other than the forestomach findings were noted at the 6000 and 18000 ppm dietary levels. These consisted of low bodyweight gain and initial reduced food consumption in both sexes. Although the bodyweight changes at both 6000 and 18000 were considered to be treatment-related, at 6000 ppm based on a combination of the short duration, the magnitude of difference from controls, lack of adverse effects on survival or general condition of the animals, they were not considered to be of toxicological importance.

In animals receiving 18000 ppm there were more pronounced effects on bodyweight gain and irritant effects of treatment on the forestomach with limited ulceration and signs of healing.

No significant toxicological effects were observed in the animals receiving 6000 ppm or 2000 ppm lauric arginate in the diet.

Based on the calculated intake data, the NOAEL in this study was 6000 ppm equivalent to 307 mg/kg bw in the males and 393 mg/kg bw in the females. The corresponding Lowest-Observed-Adverse-Effect Level (LOAEL) was 18000 ppm equivalent to 907 mg/kg bw and 1128 mg/kg bw for the males and females respectively, based on local irritant changes in the forestomach.

Oncogenicity

The rapid metabolism of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl to natural compounds found in the diet, the absence of significant systemic toxic effects in the studies performed, and the minor nature of the findings in the clinical human studies, together with the absence of mutagenic activity indicates the product is not carcinogenic and it is therefore not necessary to perform carcinogenicity studies.

Reproductive and Developmental Toxicity

The studies reviewed in this section are classified in two groups:

1. One group corresponds to a set of studies performed on a single generation of animals. In this case, the potential toxicity of lauric arginate was assessed in mothers (i.e., the reproductive behaviour and pregnancy development) and foetuses (i.e., their development and size of litters).
2. The other group consists of a preliminary study and a two generation multi-generation study.

The one generation studies used two species, rats and rabbits. In each case, two preliminary experiments were performed to establish suitable dosages of lauric arginate, administered by oral gavage, for use in the main reproductive studies. The first experiments, dose range finding studies, determined the tolerance of rats and rabbits to lauric arginate. In these experiments, the tolerance to lauric arginate of non pregnant animals and pregnant animals was compared. Further dose ranging (preliminary) studies were then performed in pregnant rats and rabbits, to select suitable doses of lauric arginate for the main studies. Finally, the third experiments (main studies) focussed on the embryo-foetal toxicity in rats and rabbits in order to evaluate the effects of lauric arginate when it was administered during organogenesis and foetogenesis and also to assess the survival and development of the unborn foetuses.

The two generation studies were performed in rats. In this case two studies were performed. First of all, a preliminary experiment was performed to find a suitable dietary concentration of lauric arginate to perform the main two-generation reproductive study. The levels of lauric arginate chosen for the dietary concentration of preliminary study were based on the results obtained in the 13-week dietary administration toxicity study of lauric arginate in rats (Appendix 10). The second study was the main reproductive two generation study and the objective was to assess the influence of lauric arginate, when administered continuously in the diet through two successive generations of CD rats on reproductive performance.

One Generation Studies:

Preliminary study of embryo-foetal toxicity in the CD rat by oral gavage administration *(Study LMA013/980140, Huntingdon Life Science, Huntingdon, UK, 1998)***.**

Introduction:

The objective of this study (Appendix 13) was to establish a suitable dose of lauric arginate, for the main embryo-foetal toxicity study in rats. The dosages selected for this study were based on a dose range finding study performed in non-pregnant and pregnant rats given lauric arginate daily for 2 or 7 days to establish the maximum

tolerated dose for use in a preliminary study of embryo-foetal development (LMA011/980114, [Appendix 12]).

As treatment of pregnant rats with 2000 mg/kg bw/day for 7 consecutive days did not produce any significant changes in maternal body weight or embryo survival, dosages of 200, 600 and 2000 mg/kg bw/day of the test substance were selected. The duration of treatment was 14 days to encompass the period of organogenesis and foetal development. Dosages higher than 2000 mg/kg bw/day of test substance were not administered as this is the maximum practical dose administered to rats on a daily basis without exceeding guideline figures for volume dosage.

As this was a preliminary study an official guideline and methodology was not followed. The study was conducted in compliance with the EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal Management: Adult virgin female Sprague-Dawley (Crl:CD®(SD)BR) rats obtained from Charles River, Margate, Kent, UK were used. An acclimatisation period of 10 days was allowed before starting any experimental work. Animals were housed 4 (during acclimatisation), 1 male and 1 female (mating phase) and 1 (gestation)/cage with food and water available *ad libitum*. Room temperature was 18-25ºC, relative humidity 40 to 70%, there were approximately 15 air changes/hour and a 12 hour artificial light/dark cycle. At the start of treatment animals were aged 10-11 weeks and within the bodyweight range 196-269 g.

Three groups of 6 female animals received the test substance by oral gavage at dosages of 200, 600 or 2000 mg/kg bw/day for 14 days (days 6-19 of pregnancy), at a volume-dosage of 10 ml/kg bw. An additional group of 6 animals (the control group) received vehicle, 1% w/v aqueous methylcellulose, alone at the same volume-dosage.

Mating procedure:

Female rats were paired on a one-to-one basis with stock males of the same strain. The day on which a sperm positive vaginal smear or at least three copulation plugs were found was designated as day 0 of gestation. Females showing unequivocal evidence of mating were allocated to group and cage position in sequence, thus ensuring that animals mated on any one day were evenly distributed amongst the groups.

Test substance:

The test substance was LAE Batch 5159 (purity 69.1%[31]).

Test substance formulation:

The test substance as supplied was formulated freshly each day in 1% w/v aqueous methylcellulose. The mixing, stability and concentration of the test substance in the formulations prepared for the low and high dosage groups were determined prior to the study start. The content of each formulation prepared for 1 day in the first and last weeks of treatment were also analysed. The results confirmed acceptable stability, homogeneity and content.

[31] The purity of this batch does not correspond to the specification for lauric arginate. LAE Batch 5159 was obtained following the same synthesis process described in Confidential Appendix 31, but using a different filter press. The only difference from lauric arginate as specified is a difference in water content.

Observations and terminal investigations:

All animals were observed at least twice daily for clinical signs and mortality. Body weight was recorded on days 0, 3 and 6-20 inclusive after mating. Food consumption was measured for days 0-2, 3-5, 6-8, 9-11, 12-15, 16-17 and 18-19 after mating. On day 20 of gestation, the animals were killed, examined macroscopically and abnormal tissues retained. The reproductive tract, complete with ovaries, was dissected out and the number of corpora lutea in each ovary, implantation sites, resorption sites and number and distribution of foetuses were recorded. All foetuses were weighed, sexed and examined for external abnormalities. Individual placental weights and abnormalities were recorded. The neck, thoracic and abdominal cavities of approximately 50% of each litter were dissected and examined. All abnormalities were recorded and the foetuses eviscerated and fixed in IMS. The remaining foetuses were preserved in Bouin's fixative.

Results:

Maternal responses:

General conditions and mortality: One female receiving 200 mg/kg bw/day of test substance was killed *in extremis* on day 19 of gestation after reduced food intake on Days 18-19 of gestation (only 2 g/day) and body weight loss of 40 g from day 18 to 19. This female had signs of pallor, piloerection, brown staining around the left orbit, red urine and a perigenital discharge at despatch. Necropsy revealed a large amount of dark red fluid within the vagina and both uterine horns. The uterus contained 15 late resorptions. The general condition of the remaining animals was satisfactory and 21 of the 23 surviving females were pregnant. One female in each treated group had periods when respiration sounded noisy. Salivation after dosing was occasionally seen in 3 of the 6 animals receiving 600 mg/kg bw/day of test substance and on about 50% of occasions in all animals receiving 2000 mg/kg bw/day of test substance.

There were no other significant clinical signs recorded in either the control or any of the treatment groups.

Bodyweight: There were no intergroup differences in bodyweight or bodyweight gain, which were considered to be treatment-related. Occasional animals showed slight body weight loss during the first two days of treatment with the test substance. However, this was related to animals adapting to the gavage dosing process rather than to the test material itself.

Food consumption: Food consumption was similar for all groups of rats throughout the treatment period.

Necropsy findings: There were no necropsy findings, which were considered to be related to treatment.

Litter responses: One control female (F-1) had only a single implantation site. Two more rats from groups 2 and 4 receiving 200 mg/kg bw/day and 2000 mg/kg bw/day respectivelyhad high pre-implantation losses. However, these losses almost certainly occurred before the start of treatment, so these individual incidences were considered not to be treatment related.

The group mean value for post-implantation loss was slightly higher in animals receiving 600 mg/kg bw/day than other groups but, in the absence of similar effects in the highest dosage group, this was considered to not be related to treatment.

Foetal examination at necropsy: There were no obvious treatment related effects upon foetal development as assessed by foetal weight and macroscopic examination at necropsy.

Conclusions:

There were no significant treatment related effects on females receiving test substance at 200, 600 or 2000 mg/kg bw/day, from day 6 of gestation until just before the expected time of parturition, or upon the survival or development of their foetuses.

Salivation seen after dosing, particularly in the highest dosage group, is a common non-specific response to gavage treatment, which may relate to the taste or pH of the test material. It usually has no toxicological significance.

2000 mg/kg bw/day of the test substance was considered to be suitable as the highest dose level for the main embryo-foetal study in the rat. This dosage was considered to be the maximum achievable dosage in relation to formulation and volume dosage under the condition of use.

Study of embryo-foetal toxicity in the CD rat by oral gavage administration *(Study LMA014/984183, Huntingdon Life Science, H*untingdon, UK, 1998).

Introduction:

The objective of this study was to assess the effects of lauric arginate when administered during the organogenesis phase of gestation until the completion of foetal development in pregnant rats and on the survival and development of the unborn foetuses (Appendix 14).

The dosages selected for this study (200, 600 and 2000 mg/kg bw/day) were based on the results obtained in the previously described dose ranging study (LMA013/980140) (Appendix 13). The dosing period was 14 days (days 6-19 of pregnancy inclusive). The study was terminated on day 20. The study was conducted in accordance with the requirements or guidelines issued by the EPA (FIFRA), EU, OECD and J-MAFF current at the time though the treatment period was extended to the end of pregnancy in anticipation of the changes set out in the revised drafts of the regulations.

The study was conducted in compliance with the EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal Management: Adult virgin female Sprague-Dawley (Crl:CD®(SD)BR) rats obtained from Charles River, Margate, Kent, UK were used and mated with stock males from the same strain and source. An acclimatisation period of 5 days was allowed before starting any experimental work. Animals were housed 4 (during acclimatisation), 1 male and 1 female (mating phase) and 1 (gestation)/cage with food and water available *ad libitum*. Room temperature was 19-23ºC, relative humidity 40 to 70%, with approximately 15 air changes/hour and a 12 hour artificial light/dark cycle in the room. At the start of treatment, animals were aged approximately 10-11 weeks and within the bodyweight range 217-267 g.

Three groups of 22 female animals received the test substance by oral gavage at dosages of 200, 600 or 2000 mg/kg bw/day for 14 days (days 6-19), at a volume-dosage of 10 ml/kg bw, based upon the most recently recorded body weight. An additional group of 22 animals (control group) received vehicle, 1% w/v aqueous methylcellulose, alone at the same volume-dosage.

Mating procedure:

Female rats were paired on a one-to-one basis with stock males of the same strain. The day on which a sperm positive vaginal smear or at least three copulation plugs were found was designated as day 0 of gestation.

Females showing unequivocal evidence of mating were allocated to a group and cage position in sequence, thus ensuring that animals mated on any one day were evenly distributed amongst the groups.

Test substance:

The test substance was LAE Batch 5159 (purity 69.1%[32]).

Test substance formulation:

The test substance as supplied was formulated freshly each day in 1% w/v aqueous methylcellulose. The mixing, stability and concentration of the test substance in the formulations prepared for the low and high dosage groups were determined prior to the study start. The content of each formulation prepared for 1 day in the first and last weeks of treatment were also analysed. The results confirmed acceptable stability, homogeneity and content.

Observations and terminal investigations:

Rats were observed at least twice daily throughout the study for visible signs of reaction to treatment and mortality. Females were weighed on Days 0, 3 and 6 to 20 inclusive after mating. Food consumption was recorded for days 0-2, 3-5, 6-8, 9-11, 12-15, 16-17 and 18-19 after mating. On day 20 of gestation, females were killed (by carbon dioxide) and their uterine contents examined. Each animal was initially examined macroscopically for evidence of disease or adverse reaction to treatment and specimens of abnormal tissues were retained. The reproductive tract, complete with ovaries, was dissected out and the following were recorded:

- the weight of the gravid uterus;
- the number of corpora lutea in each ovary,
- the number of implantation sites;
- the number of resorption sites;
- the number and distribution of foetuses in each uterine horn.

Each foetus was weighed, sexed and examined for any external abnormalities. Individual placenta weights and placental abnormalities were recorded. The neck, thoracic and abdominal cavities of approximately half of each litter were dissected and examined. All foetal abnormalities were recorded and the offspring eviscerated and fixed in methylated spirits prior to staining with Alizarin Red for skeletal examination.

The remaining foetuses in each litter were fixed in Bouin's fluid and examined by Wilson's freehand slicing.

Results:

Clinical signs and mortality: Three females receiving 2000 mg/kg bw/day of test substance (F68, F77 and F87) were killed *in extremis* on Days 7 or 8 of gestation (the second or third day of treatment). All three animals had noisy and gasping respiration, and salivation after dosing. Two of these females (F77 and F87) lost bodyweight before termination. One female (F87) was underactive and had piloerection.

[32] The purity of this batch does not correspond to the specification for lauric arginate. LAE Batch 5159 was obtained following the same synthesis process described in Confidential Appendix 31, but using a different filter press. The only difference from lauric arginate as specified is a difference in water content.

Necropsy revealed large amounts of gaseous material in the stomach of the three rats. The entire gastro-intestinal tract was distended with gas. In addition F68 had enlarged and prominent lymph nodes, and F77 had haemorrhagic lungs, large amounts of pale yellow viscous material in the ileum, reduced and dehydrated faecal contents, dark and enlarged adrenals and a pronounced internal structure of the kidneys. All animals were pregnant.

Two females at 600 mg/kg bw/day of test substance (F54, F59) were similarly affected towards the end of gestation, both had noisy respiration, salivation at the time of dosing and body weight losses. One of them (F54) was killed for humane reasons and the other one (F59) was killed *in extremis*, both had reached day 17 of gestation. Necropsy of these animals revealed that the gastro-intestinal tract was distended with gaseous material. Both animals had grossly normal implantations.

The general condition of the surviving rats was satisfactory and all the females were pregnant. Noisy respiration occurred during the treatment period in three animals receiving 200 mg/kg bw/day of test substance, a total of 7 rats at 600, and in 9 rats at 2000 mg/kg bw/day of test substance (including animals which were killed prematurely).

Salivation at the time of dosing was seen in all animals receiving 2000 mg/kg bw/day of test substance on approximately 50% of dosing occasions, reaching peak daily incidence at about day 14 of gestation. Fourteen animals receiving 600 mg/kg bw/day of test substance occasionally salivated during the dosing period, and at 200 mg/kg bw/day of test substance salivation was seen in only one animal on one occasion.

Neither noisy respiration nor salivation was seen in the Control Group.

Bodyweight: Overall there were no observed effects upon body weight related to the treatment. A few rats of all treated groups showed transient body weight losses for periods following commencement of treatment on day 6 and some animals receiving 600 mg/kg bw/day of test substance lost weight towards the end of the treatment period. Loss of weight coincided with episodes of respiratory distress. In the control groups, there were no similar bodyweight losses.

Food consumption: There were no overall treatment related effects upon food consumption with group mean values similar for all groups. However, food intake, which appeared to be associated with episodes of respiratory distress, was reduced in occasional animals in the treatment groups.

Necropsy findings at day 20 of gestation: There were no maternal necropsy findings that were considered to be related to treatment.

Litter responses: There were no effects on foetal survival as indicated by the extent of pre- and post-implantation loss and the numbers of live foetuses. In the control, low, mid and high dose groups the preimplantation losses were 5.5%, 6.7%, 7.9% and 5.5% respectively, and the postimplantation losses were 6.7%, 3.4%, 3.8% and 4.1%. Litter sizes (live foetuses) in the controls to high dose groups respectively were 14.0, 14.0, 14.1, and 14.7.

Foetal evaluation: Foetal and placental weights were unaffected by treatment. The incidences and types of major foetal abnormalities were unaffected by treatment with incidences in the controls to high dose groups respectively of 2/307; 2/309; 0/282 and 4/280. The numbers of skeletal and visceral minor abnormalities and variants were unaffected by treatment.

Conclusions:

Severe respiratory distress was recorded at a low frequency among animals receiving 600 or 2000 mg/kg bw/day of test substance. Three female rats had to be killed after 2 or 3 days of dosing at 2000 mg/kg bw/day of test substance and two rats had to be killed after 11 or 12 doses at 600 mg/kg bw/day. However, with the exception of F77, given 2000 mg/kg/day, which had haemorrhagic lungs, necropsy of these rats did not detect damage to the lungs and gross changes were limited to accumulation of gas within the GIT.

It was concluded the effects were most probably related to gasping respiration following aspiration of the irritating dosing material, especially following treatment with the more concentrated/viscous suspensions at the higher doses.

With the exception of transient effects on bodyweight and food consumption associated with individual animals showing respiratory distress, at 600 and 2000 mg/kg bw/day, there were no adverse effects of treatment on the mother and there were no adverse effects on embryo foetal survival and development at dosages of up to 2000 mg/kg bw/day.

The No-Adverse-Effect Level (NOAEL) for the dam was 200 mg/kg bw/day (based on respiratory distress associated with the irritating nature of the test material) and for foetuses was 2000 mg/kg bw/day (the highest dose tested).

Preliminary study of embryo-foetal toxicity in the rabbit by oral gavage administration *(Study LMA 015/980169, Huntingdon Life Science, Huntingdon, UK, 1998)**.***

Introduction:

This study was performed to assess the effects of repeated oral administration of lauric arginate upon the progress and outcome of pregnancy in rabbits in order to establish suitable dosages for the main embryo-foetal toxicity study (Appendix 52).

Dosages of 250, 500 and 1000 mg/kg bw/day were selected on the basis of the results of the tolerance study performed in non-pregnant and pregnant rabbits for 2-7 days, to establish the maximum tolerated dose for use in a preliminary study of embryo-foetal development (Appendix 14). Treatment at 1000 mg/kg bw/day for 7 consecutive days did not have any significant effect on embryo survival and there was no evidence of toxicity in the dams that would have precluded further investigations at this dosage. The 14-day dosing period was chosen to encompass the period of major organogenesis.

As it was a preliminary study, an official guideline and method was not followed. It was conducted in compliance with the principles of GLP though no specific study inspections were performed.

Experimental Procedure:

Animal Management: Time mated (after pairing with proven males of the same stock), sexually mature female New Zealand White rabbits obtained from Charles River, Margate, Kent, UK were used. An acclimatisation period of 5 days was allowed before starting any experimental work. Animals were housed singly with food and water available *ad libitum*. Room temperature was 15-25.5ºC, relative humidity 58 to 70% and a 14 hour artificial light/10 hour dark cycle was maintained. On arrival, animals were aged approximately 18-26 weeks and within the bodyweight range 3.11-4.26 kg at the start of dosing.

Three groups of 4 female animals received the test substance by oral gavage at a volume-dosage of 5 ml/kg bw, based upon the most recent body weight. Dosages of 250, 500 and 1000 mg/kg bw/day were administered for 14 days (days 6-19 of gestation). A 4th group of 6 females (control group) received the vehicle (1% w/v aqueous methylcellulose) only at the same volume-dosage for the same period. Animals were killed on day 29 of gestation.

Mating Procedure: Females were time-mated with New Zealand White males of established fertility at Charles River U.K. Ltd. The day of mating was designated as day 0 of gestation.

Test substance:

The test substance was LAE Batch 5159 (purity 69.1%[33]).

Test substance formulation:

The test substance as supplied was formulated freshly each day in 1% w/v aqueous methylcellulose. The mixing, stability and concentration of the test substance in the formulations prepared for the low and high dosage groups were determined prior to the study start (LMA013/980140) (Appendix 13).

The content of each formulation prepared for 1 day in the first and last weeks of treatment was also analysed. The results confirmed acceptable stability, homogeneity and content.

Observations and terminal investigations:

Maternal observations: Rabbits were observed at least twice daily throughout the study for mortality and signs of ill health. Animals were weighed daily and values were reported for days 1, 3, 6, 8, 10, 12, 14, 16, 18, 20, 24 and 28 after mating. Food consumption was measured for days 1-5, 6-12, 13-19, 20-23 and 24-28 inclusive after mating. On day 29, the day of presumed gestation, females were killed for examination of their uterine contents. Each rabbit was examined macroscopically and specimens of abnormal tissues were retained. The reproductive tract, complete with ovaries, was dissected out and the weight of the gravid uterus, number of corpora lutea in each ovary, number of implantations sites, number of resorptions sites, number and distribution of live and dead foetuses in each uterine horn recorded.

Foetal examinations: At necropsy all foetuses were weighed, sexed and examined for any external abnormalities. Individual placental weights and placental abnormalities were recorded. All foetuses were killed by subcutaneous injection of pentobarbitone sodium. The neck, thoracic and abdominal cavities of all foetuses from each litter were dissected and examined, and the sex of each foetus recorded and position within litter recorded. Following the examination, the foetuses were eviscerated and one third of the foetuses in each litter were decapitated and the heads fixed in Bouin's fluid. Torsos and the remaining intact foetuses were fixed in industrial methylated spirits.

Results:

Maternal responses:

[33] The purity of this batch does not correspond to the specification for lauric arginate. LAE Batch 5159 was obtained following the same synthesis process described in Confidential Appendix 31, but using a different filter press. The only difference from lauric arginate as specified is a difference in water content.

General conditions and mortality: All rabbits were pregnant and had live foetuses at necropsy on day 29 of gestation. One rabbit at 250 mg/kg bw/day and another at 1000 mg/kg bw/day had periods when respiration was noisy and/or slow. This response was not dosage related.

Bodyweight: Before dosing (days 1- 6) all animals gained weight satisfactorily. Between day 6 to day 12 of gestation (first week of treatment), some small losses in bodyweight of 2/6 rabbits of Control Group, 1/4 rabbits at 250 mg/kg bw/day, 2/4 at 500 mg/kg bw/day and 3/4 rabbits at 1000 mg/kg bw/day were observed. Thereafter, most animals gained weight, and by day 28 of pregnancy group mean body weight gains were similar in the control and low dose group and only marginally lower in the intermediate and high dose groups. After adjusting the body weight gains for the weight of the uterus, no intergroup differences were observed.

Food consumption: Food consumption by animals receiving 1000 mg/kg bw/day was lower than the Control Group during Days 6 – 12 of gestation and was slightly low for the rest of the treatment period. The intake was slightly depressed for females receiving 500 mg/kg bw/day throughout treatment. In the first four days after treatment stopped, animals that had received 500 or 1000 mg/kg bw/day showed increased food consumption relative to consumption during the treatment period.

Necropsy findings: There were no necropsy findings that were considered to be related to treatment for the treated females at the end of pregnancy.

Litter responses: No apparent treatment related effects on embryo-foetal survival were observed. The numbers of corpora lutea were essentially similar in all groups but intergroup variation in pre-implantation loss (occurring before treatment started) and slightly high levels of post-implantation loss at 500 mg/kg bw/day resulted in considerable differences in mean live litter size. Overall foetal weight was lowest at 1000 mg/kg bw/day but this was largely attributable to the effect of larger litter size in this group and there was no indication that the ability of the dam to support a litter was impaired by treatment.

Foetal examination at necropsy: There was a low incidence of foetal anomalies seen at necropsy but no indication of any treatment related adverse effect.

Conclusions:

Two animals from groups receiving the test substances at 250 and 1000 mg/kg bw/day suffered periods of respiratory distress during the treatment phase of the study. Reduced food consumption during the administration of the test substance at 500 and 1000 mg/kg bw/day led to slight losses in the bodyweight and this is the only effect considered to be clearly related to treatment.

250 mg/kg bw/day was considered to be the No Adverse Effect Level (NOAEL) for the mother (based on respiratory distress). Higher dosages were associated with reduced food consumption and consequently a reduced bodyweight gain. For foetuses, the NOAEL was 1000 mg/kg bw/day. A dosage of up to 1000 mg/kg bw/day of the test substance was considered to be suitable as the highest dosage level for a main embryo-foetal study in the rabbit.

Study of embryo-foetal toxicity in the rabbit by oral gavage administration *(Study LMA 016/992096, Huntingdon Life Science, Huntingdon, UK, 1999)**.***

Introduction:

The objective of this study was to assess the effects of oral administration of lauric arginate when administered during the organogenesis phase of gestation in the rabbit and on the survival and development of the unborn foetus (Appendix 53).

In the previous study (LMA 015/980169) (Appendix 52), the 500 and 1000 mg/kg bw/day dosages of test substance had adverse effects upon the mother, but did not affect the survival or development of foetuses. Taking these results into account, the dosages selected were 100, 300 and 1000 mg/kg bw/day.

The study was conducted in accordance with the requirements or guidelines issued by the EPA (FIFRA), EU, OECD and J-MAFF current at the time and in compliance with the EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal Management:

Time mated (after pairing with proven males of the same stock), sexually mature female New Zealand White rabbits obtained from Charles River, Margate, Kent, UK were used. An acclimatisation period of up to 5 days was allowed before starting any experimental work. Animals were housed singly with food and water available *ad libitum*. Room temperature was 15-25ºC, relative humidity 58 to 70% and a 14 hour artificial light/10 hour dark cycle was maintained. On arrival, animals were aged approximately 18-26 weeks and within the bodyweight range 2.94-4.34 Kg at the start of dosing.

Three groups of 22 female animals (excluding 2 animals in the 1000 mg/kg bw/day group replaced on day 7 of gestation due to difficulties in dosing) received the test substance by oral gavage at a volume-dosage of 5 ml/kg bw, based upon the most recent body weight. Dosages of 100, 300 and 1000 mg/kg bw/day were administered for 14 days (days 6-19 of gestation). A forth group of 22 females (control group) received the vehicle (1% w/v aqueous methylcellulose) at the same volume-dosage for the same period.

Animals were killed on day 29 of gestation.

Mating procedure:

Females were time-mated with New Zealand White males of established fertility at Charles River U.K. Ltd. The day of mating was designed as day 0 of gestation.

Test substance:

The test substance was LAE Batch 5159 (purity 69.1%[34]).

Test substance formulation:

The test substance was formulated freshly each day in 1% w/v aqueous methylcellulose. The mixing, stability and concentration of the test substance in the formulations prepared for the low and high dosage groups were determined prior to the study start (LMA013/980140) (Appendix 13). The content of each formulation prepared for 1 day in the first and last weeks of treatment were also analysed. The results confirmed acceptable stability, homogeneity and content.

[34] The purity of this batch does not correspond to the specification for lauric arginate. LAE Batch 5159 was obtained following the same synthesis process described in Confidential Appendix 31, but using a different filter press. The only difference from lauric arginate as specified is a difference in water content.

Observations and terminal investigations:

Maternal observations: All animals were observed at least twice daily throughout the study for signs of reaction to treatment and mortality. Females were weighed daily throughout the study and bodyweights were reported for days 0, 6, 8, 10, 12, 14, 16, 18, 20, 24 and 28 of gestation. Food consumption was measured for days 1-5, 6-12, 13-19, 20-23 and 24-28 inclusive after mating. On day 29 of presumed gestation, females were killed for examination of their uterine contents. Each animal was first examined macroscopically and specimens of abnormal tissues were retained. The reproductive tract was dissected out and the weight of the gravid uterus, number of corpora lutea in each ovary, number of implantation sites were recorded, number of resorption sites, and number and distribution of live and dead foetuses in each uterine horn were recorded.

Foetal examinations: At necropsy each foetus was weighed and examined for any external abnormalities. Individual placental weights and placental abnormalities were recorded. All foetuses were killed by subcutaneous injection of pentobarbitone sodium. The neck, thoracic and abdominal cavities were dissected and examined, and the sex of each foetus and its position within the litter recorded. The foetuses were then eviscerated and one third of the foetuses in each litter decapitated, the heads fixed in Bouin's fluid, sectioned and examined. Torsos and the remaining intact foetuses were fixed in industrial methylated spirits for subsequent skeletal examination. Eviscerated foetuses were processed using a modified Dawson Alizarin Red technique and the skeletons examined for skeletal development and abnormalities.

Results:

Maternal responses:

Clinical signs, mortality and abortion: Prior to treatment commencing all animals were healthy and gained weight satisfactorily. At 1000 mg/kg bw/day one animal (F71) was killed for humane reasons on day 9 of gestation following periods of noisy respiration accompanied by reduced food consumption and faecal output and an aqueous discharge in the cage under tray on day 9 of gestation. Necropsy revealed a small amount of frothy liquid in the trachea, and congestion in the lungs. One animal at 300 mg/kg bw/day (F60) was also killed for humane reasons on day 14 of gestation because of gasping respiration following dosing. Necropsy revealed incomplete collapse of the lungs, with occasional dark areas of change on the lung surfaces. One female at 1000 mg/kg bw/day (F84) aborted on day 24 of gestation: necropsy revealed three empty implantation sites in the left uterine horn but no implantations in the right horn of the uterus. Two dead foetuses, both of which appeared grossly normal, were found in the under tray of the cage.

Reactions to dosing were largely limited to changes in respiration pattern seen in 5 animals at 300 mg/kg bw/day and 5 animals at 1000 mg/kg bw/day (including the two animals, which were killed early in the study and replaced). Adverse respiratory reactions were believed to be associated with a higher risk of irritation being induced during the dosing procedure when high concentrations of test material were used; difficulties with dosing were much reduced when the surface of the catheter was washed clean rather than wiped dry before dose administration. There were no other signs of adverse reaction to treatment.

Bodyweight: Bodyweight gain of animals receiving test substance at 1000 mg/kg bw/day was slightly but significantly lower than that of the Controls throughout most of the treatment period. This was considered to be treatment related, because the animals showed recovery of weight gain once treatment was completed, although interpretation was complicated by the fact that animals allocated to the high dosage group had gained more body weight than Controls in the period between mating and start of treatment.

Bodyweight gains of animals receiving test substance at 100 or 300 mg/kg bw/day were similar to that of the Controls throughout gestation.

Food consumption: Food consumption by animals receiving the test substance at 1000 mg/kg bw/day fell slightly when treatment started and was significantly lower than that of the Control group during Days 13-19 of gestation but recovered to similar to Control levels after completion of the dosing period. Food consumption of test substance at 100 and 300 mg/kg bw/day was unaffected by treatment.

Necropsy findings: There were no necropsy findings for females killed on day 29 after mating that were considered to be related to treatment with test substance.

Litter responses: There were no apparent treatment related effects on foetal survival. The numbers of corpora lutea implantations and live young in the control group were generally lower than in the treated groups but intergroup differences were not statistically significant. In the Control, low, mid and high dose groups respectively, the pre-implantation losses were 8.9%, 13.4%, 12.3% and 11.3%, and the post-implantation losses were 9.3%, 11.9%, 10.3% and 6.9%. Litter sizes (live foetuses) in the controls to high dose groups respectively were 8.9, 9.1, 9.1 and 10.0.

Foetal examination at necropsy: There were no effects of treatment on foetal weight or placental weight. The incidences and types of major foetal abnormalities were unaffected by the treatment with incidences in the controls to high dose groups respectively of 3/169; 4/200; 3/163 and 2/170. The numbers of skeletal and visceral minor abnormalities and variants were unaffected by the treatment.

Conclusions:

Treatment of rabbits with the test substance by oral gavage at 300 or 1000 mg/kg bw/day was associated with difficulty in dosing and signs of respiratory distress in some animals. Similar respiratory signs were recorded during a preliminary study (Appendix 52) and also in a rat study (Appendix 14). This clinical sign was related to aspiration of traces of the test material. However, altering the standard dosing procedure, by using a clean moist catheter instead of a clean dry catheter, appeared to alleviate some of the dosing problems although there was still a residual incidence of respiratory noises.

Treatment at 1000 mg/kg bw/day was associated with reduced maternal bodyweight gain during treatment and also with reduced food intake. In contrast, these effects were not observed at 100 and 300 mg/kg bw/day. There were no adverse effects upon foetal survival and development at any dosages up to the maximum level tested (1000 mg/kg bw/day).

Despite the slightly higher risk of irritation to the respiratory tract at dosages of 300 mg/kg bw/day and above, it was concluded that 300 mg/kg bw/day of the test substance was the No-Adverse-Effect Level (NOAEL) for the dam and 1000 mg/kg bw/day of the test substance was the NOAEL for the foetus.

Two Generation Study:

Preliminary study of effects on reproductive performance in CD rats by dietary administration *(Study LMA 041/032575, Huntingdon Life Science, Huntingdon, UK, 2003).*

Introduction:

This study examined two generations of rats (F0: parental generation and F1: first generation) when they were exposed to lauric arginate (Appendix 54). The effects of continuous exposure to lauric arginate in the diet were assessed across the following periods:

- reproductive,
- pregnancy and
- development of offspring.

The dietary concentrations were selected based on toxicological data available from a 13-week dietary study in Han Wistar rats (Appendix 10) in which a concentration of 5000 ppm of lauric arginate (384 and 445 mg/kg bw/day for male and female rats respectively) was identified as the No-Observed-Adverse-Effect Level (NOAEL) and the NOAEL for dams and foetuses which was established as 200 and 2000 mg/kg bw/day respectively in the study of embryo-foetal toxicity in rats (Appendix 14).

Lauric arginate was administered daily in the diet instead of by oral gavage in order to avoid the respiratory irritation observed in some rats and rabbits in the one generation studies previously performed.

The data generated in this study were used to assist with the selection of the dietary concentrations for a subsequent two-generation study.

This study was conducted in compliance with the EU, OECD and UK GLP Guidelines

Experimental Procedure:

Animal Management:
F0 Generation: Adult male and virgin female Sprague-Dawley (Crl:CD®(SD)IGSBR(IGS)) rats obtained from Charles River, Margate, Kent, UK were used. An acclimatisation period of 5 days was allowed before starting any experimental work. Animals were housed 4 (during acclimatisation and males after mating), 1 male and 1 female (premating and mating phase and 1 female (gestation)/cage with food and water available *ad libitum*. Room temperature was 20-23ºC, relative humidity 38 to 53%, approximately 15 air changes/hour and a 12 hour artificial light/dark cycle was maintained. At the start of treatment, animals were aged approximately 9-10 weeks and within the bodyweight range 308-343g (males) and 194-217g (females).

Three groups of 8 male and 8 female animals received lauric arginate continuously via the diet at concentrations of 1500, 5000 or 15000 ppm for 4 weeks before pairing, throughout mating and until termination after weaning of the litters. An additional group of 8 male and 8 female animals received diet alone and acted as a control group.

F1 Generation: The F1 generation was constituted of 4 groups of 12 male and 12 female offspring of the F0 animals. Following weaning, a minimum of one male and one female offspring, with additional offspring of a maximum of two males and two females per litter from as many F1 litters as possible in each group were selected to form the F1 generation. Animals were housed 4/sex/cage after weaning in the same conditions as the F0 generation. Treatment was continued at the same dietary

concentrations as the F0 generation had received previously from weaning until termination.

Mating procedure:

F0 females were paired on one-to-one basis with males from the same treatment group for a maximum of two weeks. The day on which mating was detected was designated as day 0 of gestation. Once mating had occurred, the males and females were separated and vaginal smearing discontinued.

Test substance:

The test substance was LAE Batch 10234 (purity 88.2%).

Test substance formulation:

Diets were usually prepared fortnightly. For each concentration the required amount of lauric arginate was stirred together with an approximately equal amount of basal diet. This doubling process with basal diet was repeated until a total mixture of approximately 2 kg was achieved. This premix was added to appropriate quantities of basal diet and then mixed. The suitability of the mixing procedure for lauric arginate formulations and their stability were determined. Samples of all diets prepared in week 1 of treatment (F0) and week 2 (F1) were analysed to assess concentration. The procedures, stability and achieved concentrations were satisfactory.

Observations and terminal investigations:

All animals were inspected twice daily for signs of reaction to treatment, ill health and mortality. Approximately weekly all parental and selected F1 animals were subjected to a physical examination. Males were weighed on the first day of treatment and twice weekly until termination. Females were weighed on the first day of treatment and then weekly until mating was detected. Subsequently, they were weighed on days 0, 6, 13 and 20 after mating and on days 1, 4, 7, 14 and 21 of lactation. Selected F1 rats were weighed twice weekly from a nominal 4 weeks of age until termination at approximately 8 weeks of age. Food consumption was measured twice weekly for the F0 animals until they were paired for mating. Subsequently for females, food consumption was recorded for days 0-5, 6-12, 13-19 days after mating and days 1-3, 4-6, 7-13, 14-20 during lactation. For F1 rats, food intake was measured twice weekly from nominal week 4 of age until termination.

F0 females were paired on a one-to-one basis with males from the same treatment group for a maximum of two weeks. After pairing, vaginal smears were taken daily, by vaginal lavage, from all F0 females until evidence of mating was observed. The day on which mating was detected was designated day 0 of gestation. Once mating had occurred, the males and females were separated and vaginal smearing discontinued.

The time elapsed between initial pairing and detection of mating was recorded.

From day 20 after mating, F0 females were inspected three times daily for the onset, progress and completion of parturition. All F0 females were permitted to deliver their young naturally and rear their own offspring until day 21 of lactation where possible.

Serial Observations (litters):

Twenty four hours after birth the offspring were examined. The number of offspring (live and dead), individual offspring bodyweight, sex ratio and any observations were recorded and the pups identified. Offspring were examined daily from day 1 to day 21 of age. Mortality and consequent changes in litter size on Days 1-21 of age were recorded. Litters were culled to 5 males and 5 females on day 4 of age. The sex of offspring was determined on days 1 and 4 of age and at weaning. The live offspring were weighed individually on days 1, 4, 7, 14, 21 and 25 of age, and then, twice

weekly for selected F1 males and females. Following weaning, offspring were selected to form the F1 generation.

Selected F1 females were examined daily from day 28 of age to assess vaginal opening; smears were taken from all F1 females to establish the normality or otherwise of their oestrous cycles. F1 males were examined daily from day 38 and balano-preputial separation recorded.

Terminal studies:
All adult animals, weaned animals and live neonates older than 14 days of age were killed by carbon dioxide inhalation. The offspring were culled on day 4 of age, and any that were killed for humane reasons before day 14 of age, were killed by an intraperitoneal injection of sodium pentobarbitone. All F0 animals were subjected to a detailed macroscopic examination for evidence of disease or adverse reaction to treatment. For females, the number of implantation sites was also recorded.

All selected F1 generation animals were subjected to detailed macroscopic examination for evidence of disease or adverse reaction to treatment.

Results:

Parental generation (F0): The general condition of rats receiving a diet containing lauric arginate was similar to that of the control group. No deaths occurred.

Bodyweight: Bodyweight gain of males was not adversely affected by treatment. The bodyweight gain for females before pairing was not clearly affected by treatment. Although overall gain for the treated females was lower than control values, there was no dosage dependent trend. Bodyweight gain during gestation and weight changes during lactation were similar in all groups.

Food consumption and food conversion efficiency: Food consumption and food conversion efficiency for both sexes were similar for control and treated animals.

Achieved dosage: The intake of the test substance was not constant during the treatment. Lauric arginate intake declined for male rats (25-26%) and female rats (14-19%) by the time of pairing which was consistent with the growth of the animals during the 4 weeks period. In males and females during the gestation period, intake of lauric arginate at each dose level continued to decline. In contrast, the intake increased noticeably during lactation in response to the physiological demands of the litter, reaching a level of more than twice the pre-pairing intake during the second week of lactation.

Mating performance and fertility: Pre-coital interval and fertility were unaffected by treatment with lauric arginate.

Gestation length and gestation index: Gestation length and gestation index were also unaffected by treatment at levels of up to 15000 ppm. All females had gestation lengths within the range 22 to 23 days and there were no cases of dystocia.

Litter responses: The general condition of the offspring in the treated groups was similar to that of the Control offspring.

Implantation sites, litter size and survival: The total number of implantations, total litter size and live litter size were essentially similar in all groups and were considered to be unaffected by the level of lauric arginate in the diet. At 15000 ppm two of the eight litters lost weight between Days 1 and 4 of age and were terminated on day 4 of age. There was also a small reduction in survival indices of those females rearing their young to weaning at this level. A possible association between treatment

and the slight increase in early postnatal litter loss and subsequent levels of offspring death could not be excluded.

Sex ratio: Sex ratios were unaffected by the treatment and were close to 50% in all groups.

Bodyweight: On day 1 the bodyweight of the offspring was unaffected by the presence of lauric arginate in the parental diet at concentrations of up to 15000 ppm. The bodyweight of the offspring throughout the preweaning period to day 25 of age was unaffected by treatment, either as a result of indirect exposure via the milk or direct exposures as they established independent feeding.

Terminal examinations:
F0 macropathology findings: No remarkable findings were detected in the F0 generation. Mammary tissue appeared inactive or had only small amounts of milk present for the two females terminated after their litters had died.

F1 macropathology for unselected offspring: Absence of milk in the stomach was the predominant finding at necropsy of the offspring found dead before weaning, indicating that the pup had not been fit to suckle or that the mother failed to supply sufficient milk.

At 15000 ppm, the increased incidence of pups with no milk in the stomach was considered to reflect the reduced lactation by two of the females in the early postnatal period.

F1 generation:
The general condition of selected F1 animals from the treated group was similar to that of the control. There were no significant clinical signs and no death occurred.

Bodyweight: The bodyweight of selected F1 males and females at approximately four weeks of age and gains through to approximately eight weeks of age, were similar to the respective control group values and were considered to be unaffected by treatment.

Food consumption and food conversion efficiency: Food consumption and food conversion efficiency for males and females in the treated groups was similar to that of the respective controls.

Achieved dosage: Achieved dosage for animals in the highest treatment group (15000 ppm) exceeded 2100 mg/kg bw/day of lauric arginate during the first week after selection (age 4-5 weeks) and was similar to the F0 maternal values seen during days 4-6 of the first week of lactation (i.e., 2314 mg/kg bw/day). Intake throughout the period was higher than achieved for the F0 animals, reflecting the expected pattern of the low bw/high food consumption of the young animals. The exposure level of F1 selected animals in the first two weeks after selection was approximately double the average exposure for the F0 generation during the four weeks before pairing.

Sexual maturation: The treatment with lauric arginate did not affect the balano-preputial separation.

Vaginal opening at the highest dose of lauric arginate administered in the diet (15000 ppm) happened 4 days later than in the control females. The bodyweight of these offspring at sexual maturation was higher than Controls but there was no consistent relationship between body weight and time of vaginal opening.

Oestrous cycles: Vaginal smearing was initiated at 42 days of age for all groups. The first evidence of oestrus was seen at approximately 44 days of age in all groups. The first recorded oestrous cycle in the Controls was generally of 4 days duration, the normal mature cycle length in this strain, but treated animals showed a higher proportion of 5-days cycles for the first recorded cycle. However, the subsequent cycle was reduced to 4 days in nearly all cases in all groups and it was considered that the delay in vaginal opening had no long lasting impact upon the normal sexual development of the female rats.

Terminal examinations of selected F1 animals: The necropsy of the F1 offspring at approximately 8 weeks of age detected no macroscopic changes that were considered to be related to treatment.

Conclusions:

No adverse effects were observed on bodyweight, bodyweight change, food consumption or conversion efficiency at any level of lauric arginate administered.

There were no apparent effects on mating performance, fertility or litter size at birth associated with the level of test material administered in the diet and the growth of the selected F1 offspring was satisfactory. Two of the eight litters at 15000 ppm lost weight between birth and day 4 of age; the offspring were debilitated and necropsy on day 4 of age revealed that they had no milk in their stomachs. The females rearing young to scheduled termination also showed some evidence of impaired offspring survival although this was not associated with any clear affect of growth.

In the F1 generation, vaginal opening was delayed in the 15000 ppm treatment group but this had no long term consequences on the establishment of normal oestrous regularity and no effects were observed on balano-preputial separation. However, the effects were not so marked as to preclude the selection of 15000 ppm as the highest treatment level for the main multigeneration study in the rat.

It was concluded that a dietary concentration of 15000 ppm of lauric arginate could be used as the highest treatment level for the main two-generation study in the CD rat.

Two generation reproductive performance study by dietary administration to CD rats *(Study LMA 042/032553, Huntingdon Life Science, Huntingdon, UK, 2005).*

Introduction:

The objective of this study was to assess the influence of lauric arginate on reproductive performance when administered continuously in the diet through two successive generations of CD rats (Appendix 55).

The study was conducted in accordance with the requirements of the Organisation for Economic Development (OECD) Test Guideline 416: Proposal adopted, 21 January 2001 and in compliance with the EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Animal Management:

F0 Generation: Adult male and unrelated virgin female Sprague-Dawley (Crl:CD®(SD)IGS BR) rats obtained from Charles River, Margate, Kent, UK were used. An acclimatisation period of 12 days was allowed before starting any experimental work. A routine health check was performed on 4 animals of each sex to confirm the animals were healthy. Animals were housed 4 (during acclimatisation and males after mating, females after weaning), 1 male and 1 female (premating and mating phase) and 1 female (gestation, littering lactation)/cage with food and water available *ad*

libitum. Room temperature was 19-23ºC, relative humidity 40 to 70%, with its own supply of filtered fresh air which was not recirculated and a 12 hour artificial light/dark cycle was maintained. At the start of treatment, animals were aged approximately 38-42 days and within the bodyweight range 138-250g (males) and 127-181g (females).

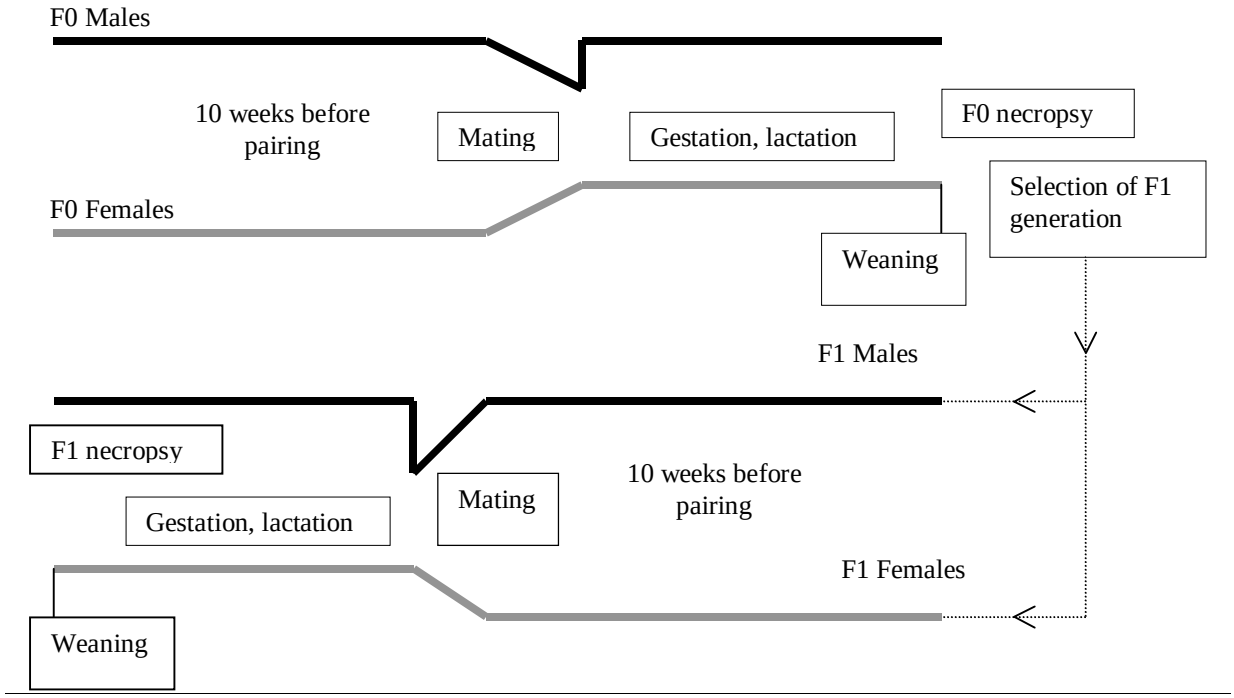
Three groups of 28 male and 28 female animals received lauric arginate continuously in the diet at concentrations of 2500, 6000 or 15000 ppm 10 weeks before pairing, throughout mating and until termination after weaning of the litters. An additional group of 28 male and 28 female animals received diet alone and acted as a control group.

F1 Generation: F1 offspring were numbered individually within each litter on day 1 of age. At 28 days of age, the rats were allocated for the study. Where possible, one offspring of each sex from each litter was selected for continuation of the study.

Each group consisted of 24 rats of each sex. The animals were housed 4/sex/cage after selection in the same conditions as described above for the F0 generation. Treatment was continued at the same dietary concentrations as the F0 generation had received previously from weaning until termination.

F2 Generation: The F2 offspring were numbered individually within each litter on day 1 of age and litter size adjusted to 5 male and 5 female offspring prior to necropsy.

The following scheme outlines the design of this study:



Test substance:

The test substance was LAE Batch 10234 (purity 88.2%).

Test substance formulation:

A series of graded concentrations of lauric arginate in the diet were prepared. The required amount was weighed and stirred together with an approximately equal amount of basal diet. This doubling process with basal diet was repeated until a total mixture of approximately 2 kg had been achieved. Portions of this premix were then

added to appropriate weights of the basal diet and then mixed. The test substance was used as supplied. Formulations were usually prepared fortnightly and stored at ambient temperature. The suitability of the mixing procedure for lauric arginate formulations and their stability were determined. Samples of all diets prepared in week 1, 11, 19, 30 and 35 of the study were analysed to assess concentration. The procedures, stability and achieved concentrations were satisfactory.

Observations and terminal investigations:

Rats were visually inspected at least once (acclimatisation) or twice per day for signs of reaction to treatment (treatment period), ill health and mortality. A physical examination to monitor general health was performed weekly.

F0 males were weighed at the start of treatment, at weekly intervals and before necropsy. F0 Females were weighed at the start of treatment, at weekly intervals until mating was detected, on days 0, 6, 13, 20 after mating, on days 1, 4, 7, 14, 21 of lactation and before necropsy (day 28 post partum).

F1 rats were weighed at the same frequency as F0 animals following selection at 4 weeks of age.

Food consumption was measured each week of treatment before animals were paired and mated. For F0 females, food consumption was measured for days 0-5, 6-12 and 13-19 after mating and days 1-3, 4-6, 7-13 and 14-20 of lactation. Following selection at 4 weeks of age the food consumption of F1 rats was measured at the same frequency as F0 animals. For 15 days before pairing of F0 and F1 females daily vaginal smears were taken. The smears were examined to establish the duration and regularity of the oestrous cycle. After pairing, smearing was continued until evidence of mating was seen. Daily vaginal smears taken from females that reared their litters to weaning were used to determine the stage of the oestrous cycle at termination.

After 10 weeks of treatment/10 weeks after selection, F0/F1 males and F0/F1 females from same treatment group were paired on a one-to-one basis for a period of up 3 weeks. The day on which evidence of mating was found was designated day 0 of gestation. Males and females were separated once evidence of mating was observed. From day 20 after mating, females were inspected 3 times per day for evidence of parturition.

Litters were examined 24 h after birth (day 1 age) and daily thereafter for clinical signs, litter size, sex ratio (days 1, 4 and 21 of age), bodyweight (days 1, 4, 7, 14, 21 and 25 of age), ano-genital distance (day 1 for all F2 offspring due to evidence of a delay in vaginal opening). Pre weaning, surface righting was assessed daily from day 1 of age until achieved, air righting was assessed daily from day 14 of age until achieved, auditory function, startle response to a sudden sharp sound, were assessed on day 20 of age as were visual function, pupil closure response of dark adapted eyes to bright point source of light.

Offspring were weaned on day 21 and were selected for the F1 generation on day 25. Sexual maturation in F1 generation males was assessed daily by examination from day 35 until balano-preputial separation. Bodyweight was recorded on day separation completed. Sexual maturation in F1 generation females was assessed daily from day 28 until vaginal opening. Bodyweight was recorded on the day of vaginal opening.

Terminal investigations:

F0 and F1 males were killed when the majority of litters had weaned after at least 17 and 16 weeks of treatment respectively. Females that littered and reared offspring to weaning were killed on day 28 *post-partum*, after their respective litters had been weaned. Females that failed to mate were killed on day 25 after the last day of pairing

while females that failed to produce a viable litter were killed day 25 after mating. Females whose litter died before day 21 of lactation were killed on or after the day the last offspring died. F1 or F2 offspring were culled on day 4 to leave 10 pups/litter. Other offspring that were not selected to form F1 generation and F2 offspring were killed on day 30.

F0/F1 generation adult animals and all offspring killed after day 14 were killed by CO_2 asphyxiation. Offspring killed before day 14 received an intraperitoneal injection of sodium pentobarbitone. F0/F1 adult rats and F1/F2 offspring were subjected to a detailed necropsy including an examination of tissues and all external features and orifices. Samples of sperm for analysis were taken from males soon after death. The neck and associated tissues, thoracic, abdominal and pelvic cavities and their viscera were examined *in situ*. The requisite organs were weighed and external and cut surfaces of organs and tissues were examined and samples preserved in appropriate fixative and submitted to a microscopic examination. For females the number of implantation sites in each uterine horn was recorded.

Only those unselected offspring culled on day 4 of age with external abnormalities were examined.

With the exception of missing, grossly autolysed or grossly cannibalised offspring, those dying prematurely before weaning and unselected offspring killed at day 30 of age were examined as above. Where possible, one male and one female offspring from each litter of the latter group were weighed and the requisite organs weighed and fixed as detailed below.

Sperm motility in all males and sperm morphology, sperm count, and homogenisation-resistant spermatid count were examined in males in the control and 15000 ppm groups.

Results:

Lauric arginate administration:

Achieved dosages F0 generation: During the first week of treatment the achieved intake, at the highest dietary concentration of 15000 ppm, was calculated to be 1635/1687 mg/kg bw/day for males and females respectively. By the time of pairing lauric arginate intake had declined by around 52% for males and 44-45% for females.

The mean achieved dosages, expressed as mg/kg bw/day, of the F0 generation are presented as Table 14 below:

Table 14: *Mean Achieved Doses*

	F0 Generation					
Dietary concentration (ppm)	**Males**			**Females**		
	2500	**6000**	**15000**	**2500**	**6000**	**15000**
Before pairing	181	434	1073	207	502	1226
During gestation	---	---	---	231	585	1518
During lactation	---	---	---	402	1018	2600

Achieved dosages F1 generation: During the first week of treatment the achieved dosage in the animals (aged 4-5 weeks) of the highest treatment group exceeded

2200 mg/kg bw/day. This intake was similar to the F0 maternal values seen during the first week of lactation. During the remaining pre-pairing period the intake was higher than that of the F0 animals reflecting the lower body weight and higher food consumption of the younger animals. At the time of pairing the intake of lauric arginate had declined about 60-62% for males and 51-54% for females, which was consistent with the growth of animals during the previous 10 weeks.

The mean achieved dosages, expressed as mg/kg bw/day, of the F1 generation are presented as in the following table:

Table 15: Mean Achieved Dosages

	F1 Generation					
Dietary concentration (ppm)	**Males**			**Females**		
	2500	**6000**	**15000**	**2500**	**6000**	**15000**
Before pairing	224	537	1356	246	582	1489
During gestation	---	---	---	215	535	1430
During lactation	---	---	---	409	898	2353

F0 Generation – in life investigations:
General condition and mortality: Animals that received a diet containing lauric arginate were generally in a similar condition to the Controls throughout. Three deaths occurred:

At 2500 ppm: 1 male was killed for humane reasons. This death was not considered to be treatment related.

At 15000 ppm: 1 male was found dead, the histopathological examination revealed a malignant nephroblastoma. 1 female was killed for humane reasons the pregnancy status was unconfirmed.

These deaths were not considered to be treatment related.

Bodyweight:
F0 males: body weight and body weight gain were not adversely affected by treatment.

F0 females: before pairing, females were not affected by the treatment. During gestation, the body weight gain of treated rats was significantly greater than control by between 11-19%. This increase was not considered to be adverse. During lactation, the body weight gain was similar in all groups.

Food consumption and food conversion efficiency:
Food consumption: was similar for control and treated animals before pairing. During gestation and lactation treated females did not show any effects of treatment.

Food conversion efficiency: by treated males and females in the pre-pairing period was similar to control animals.

Oestrous cycles pre-pairing:
There were no adverse effects of lauric arginate on oestrous cycles at dietary concentrations of up to 15000 ppm.

Mating performance and fertility:
Pre-coital interval and fertility were both unaffected by treatment. One female in each treatment group was not pregnant. This result was within background limits.

Gestation length and gestation index:
F0 animals were unaffected by treatment at levels of up to 15000 ppm. The majority of females had a gestation length between 22 to 23 days. At 2500 ppm 1 female had a longer gestation length but for the small litter size of four pups this is not unusual. There were no cases of dystocia.

F1 Offspring – pre-selection investigations:

General condition of offspring: The treated groups of F1 offspring were generally in similar condition to the Control offspring.

Litter size and offspring survival: The number of implantations, total litter size, live litter size and offspring survival were similar in all groups and were considered to be unaffected by the level of lauric arginate in the diet. Only 2 litters had any problems:

At 15000 ppm: 1 litter lost body weight between days 1 and 4 of age and was terminated on day 4 of age.

At 2500 ppm: 1 female had a total litter loss and was observed to give birth to one dead pup. This case was of no toxicological concern.

Sex ratio: Based on the percentage of male offspring the ratio was unaffected by treatment and was close to the expected value of 50% in all groups.

Offspring bodyweight: Bodyweight of offspring on day 1 was unaffected by the treatment in the parental diet at 15000 ppm. At the highest concentration, offspring body weight up to day 14, was unaffected by the treatment but a reduction in the body weight gain from day 14 was observed as the animals established independent feeding with animals being approximately 8% lighter than controls by day 25 of age.

Pre-weaning examinations: Surface righting, air righting and the auditory and visual responses of offspring were comparable in all groups and were not affected by lauric arginate in the diet.

F0 Generation/F1 Offspring – terminal investigations:

Oestrous cycles-prior to termination: Vaginal smears taken after weaning on day 25-28 post-partum showed that treatment with lauric arginate did not delay the return to a normal oestrous cycle, with all treated females showing oestrous before termination on day 28 post-partum.

Sperm assessment: At 15000 ppm a slight but non-significant reduction in the percentage of progressively motile sperm was observed.

Organ weights (F1 weanlings): *At 15000 ppm*: absolute spleen weights for F1 male and female offspring were significantly lower than control (14 and 13% less respectively).

Males had a significantly lower (11%) spleen weight relative to body weight and reduced absolute thymus weight (10% lower than control group).

At 6000 ppm: a reduction in bodyweight relative to thymus weight of males (around 7% lower than control group).

Organ weights (F0 adults): *Male rats:* after 17 weeks of treatment, absolute body weight and relative organ weights for male animals were unaffected by the treatment.

Female rats: on day 28 post-partum at 15000 ppm bodyweight relative weights of ovaries and spleen showed a statistically significant reduction when compared with controls (10 and 8% respectively).

On day 28 post-partum at 2500 ppm bodyweight relative to ovary weight showed a statistically significant reduction (10% lower than controls). However, as no dosage dependant trend was apparent, this is not considered to be toxicologically significant.

Offspring macropathology (F1): *Offspring dying before weaning:* the predominant finding at necropsy was the absence of milk in the stomach. This is not considered to be treatment related.

Offspring necropsied on day 30 of age: showed no changes attributable to treatment.

Macropathology and histopathology (F0): *F0 males after 17 weeks of treatment:* no observations or microscopic changes attributable to treatment. In particular there were no obvious effects on sperm staging.

F0 females on day 28 post-partum: no observations or microscopic changes attributable to treatment.

F1 Generation – in life investigations:
General condition and mortality: No signs observed were considered to be related to the treatment of F1 animals. Although a few animals were killed, the deaths were unrelated to treatment.

At 15000 ppm: one male and one female were killed for humane reasons after the offspring were weaned.

At 2500 ppm: one female was killed for humane reasons after the offspring were weaned.

Bodyweight: Weights of males and females at 15000 ppm were marginally lower than controls (3-5% lower, respectively) at the start of the generation reflecting reduced body weight gain after day 14 of age.

Overall bodyweight change in males was unaffected by the treatment up to termination.

Bodyweight gain during the first week at 6000 and 15000 ppm: females had a lower body weight gain than controls. These effects were only seen when the achieved dosages were at their peak >1900 mg/kg bw/day for females receiving 15000 ppm in the diet.

During late gestation, only females at 15000 ppm had a marginally low body weight change when compared with controls. This difference was made up during the latter half of lactation, when the body weight gain at 6000 and 15000 ppm was superior to that of control animals.

Food consumption and food conversion efficiency: Food consumption for animals before pairing and for females during gestation and lactation was unaffected by treatment with lauric arginate. The food conversion efficiency for the period before pairing was unaffected by treatment with lauric arginate.

Sexual maturation: *Males:* the sexual maturation, assessed by age and body weight at completion of balano-preputial separation, was unaffected by treatment.

Females: vaginal opening in animals at 15000 ppm lauric arginate was delayed by 4 days and body weight of these offspring at sexual maturation was higher than for control animals. This finding was considered related to treatment but occurred at a time when achieved dosage at 15000 ppm was calculated to be in excess of 1900 mg/kg bw/day.

At dose levels of 2500 and 6000 ppm no effects were observed on the vaginal opening of females.

Oestrous cycles- pre-mating: Prior to mating oestrus cycles were unaffected by treatment with lauric arginate at any concentration.

Mating performance and fertility: These parameters were assessed by the pre-coital interval and percentage mating. Results indicated that animals were unaffected by treatment with lauric arginate at dietary concentrations up to 15000 ppm.

Gestation length and gestation index: Both parameters were unaffected by treatment at 15000 ppm. Gestation lengths were within the range 22 to 23 days, except for 1 female in the 15000 ppm group with a gestation length of 23.5 days (for the small litter size of 3 pups this is not unusual). There were no cases of dystocia.

F2 Offspring:
General condition of offspring: The general condition of offspring in the treated groups was similar to that of control offspring.

Litter size and offspring survival: The number of implantations, total litter size, live litter size and offspring survival were generally similar in all groups and were unaffected by the level of lauric arginate in the diet.

Control group: 1 litter failed to thrive and was terminated on day 2 of age.

At 2500 and 6000 ppm: 1 litter of each group was killed for humane reasons at or immediately following weaning as most of the offspring had died and the surviving offspring were atypically small and considered unlikely to survive following the removal of the parent female.

Sex ratio and ano-genital distances: *Sex ratio*: was assessed by the percentage of male offspring. The sex ratio was unaffected by treatment and was close to the expected value of 50% in all groups.

Ano-genital distances: there were clear distinctions between males and females. The distances recorded showed no differences between treated groups and control animals.

Offspring bodyweight: *Day 1 of age:* the body weight was unaffected by the presence of lauric arginate in the parental diet at concentration of up to 15000 ppm.

Day 21 of lactation: at 15000 ppm the body weight of offspring was unaffected by the treatment.

A small but significant reduction (7%) in cumulative body weight gain was recorded for the day 1-21 period; this had resolved by day 25, with weight gains between days 21 and 25 similar to controls.

Pre-weaning examinations: Group mean values for surface righting, air righting and the visual responses of offspring were comparable in all groups and were not affected by maternal treatment. However:

At 6000 ppm: Poor performance in the startle response was observed in 2/23 litters, which had high incidence of failure.

At 15000 ppm: Poor performance in the startle response was observed in 2/24 litters with high incidences of failure.

However, as similar findings were not apparent in the first generation, the significance of this finding is uncertain.

F1 Generation/ F2 Offspring – terminal investigations:
Oestrous cycles-prior to termination: Vaginal smears taken after weaning on day 25-28 post-partum showed that treatment with lauric arginate did not delay the return to a normal oestrous cycle, with the majority of females showing oestrous before termination on day 28 post-partum.

Sperm assessment: No adverse effects on sperm motility, concentration or morphology were apparent after treatment with lauric arginate at dietary concentrations of up to 15000 ppm.

Offspring organ weights (F2 weanlings): *At 15000 ppm:* females had a significant reduction in absolute spleen weights (14%) but analysis relative to body weight was not statistically significant. Males showed a not statistically significant reduction in spleen weights of (12%).

Organ weights (F1 adults): There were no conclusive effects of treatment on organ weights. In males, absolute seminal vesicle weights were reduced (10%) after 16 weeks of treatment at the intermediate dosage of 6000 ppm. This finding was considered incidental. On day 28 post-partum, females at 15000 ppm had reduced spleen weight (9%).

Neither of these parameters showed significance with regard to bodyweight relative values.

Offspring macropathology (F2 weanlings): The F2 offspring that died prior to scheduled termination did not show any changes that could be attributed to treatment.

The macroscopic examination of F2 offspring at scheduled termination showed no effects considered to be related to treatment.

Macropathology and histopathology (F1 adults): Macroscopic examination of F1 males after 16 weeks of treatment and F1 females on day 28 post-partum did not reveal any observations that could be attributed to treatment.

There were no microscopic changes attributable to the administration of lauric arginate.

There were no effects on the ovarian follicle counts of selected F1 offspring.

Discussion and Conclusions:

Pre-pairing period:

F0 generation: the average achieved dosage exceeded 1000 mg/kg bw/day in animals that received lauric arginate at the dose level of 15000 ppm.

F1 generation: animals achieved approximately 20-30% higher intake due to exposure at a younger age when animals have high food intake relative to bodyweight.

This result is consistent with the growth rate of the animals.

Observations:

Adults: no deaths were attributable to treatment with lauric arginate in the diet up to 15000 ppm. Moreover, no adverse effects on signs, body weight performance, food consumption and food conversion efficiency were observed in adult animals.

Measures of oestrous cycles, fertility and primordial follicle counts were also unaffected by dietary exposure to lauric arginate at concentrations of up to 15000 ppm.

F1 and F2 offspring: there were no adverse effects on litter size, sex ratio, survival and day 1 bodyweight at dietary levels of up to 15000 ppm.
Bodyweight gains up to day 14 of lactation were unaffected by exposure to lauric arginate, although in the latter stages of lactation/early stages of independent feeding animals at 15000 ppm showed a slight reduction in body weight gain.

Pre-weaning examinations of surface and air righting and pupil response detected no treatment related differences.

There was a reduction in pass rate for startle response of 8.6% and 7.1% respectively for F2 offspring treated at 15000 and 6000 ppm. No similar effect had been seen in the F1 generation and no effect was seen on startle response in adult rats treated for 12 weeks at levels of up to 50000 ppm in the diet.

Balano-preputial separation was unaffected at all dosage levels.
F1: a delay of 4 days in vaginal opening at the dietary level of 15000 ppm. Females were heavier than controls at completion of the development process.

The timing of sexual maturation occured shortly after the time of highest intake (>1900 mg/kg bw/day) of lauric arginate, approximately 2 weeks after the initiation of the F1 generation.

The timing of the vaginal opening had no impact upon oestrous cycles pre-pairing or pre-termination, on fertility or on primordial follicle counts.

The percentage of males in each litter and the measurement of anogenital distance in the F2 offspring were also unaffected by treatment.

These observations indicate that lauric arginate caused no changes in sexual differentiation and that the delay in vaginal opening was of no long-term toxicological importance.

Terminal investigations:

In F0 and F1 adult animals there were no effects on sperm assessment. Macroscopic examination of adult animals and offspring revealed no changes attributable to treatment.

In the 15000 ppm group absolute and/or body weight relative spleen weights of F0 and F1 females at scheduled termination and of male and female weanlings and F2 female weanlings on day 30 of age were significantly lower than in the Controls.

The magnitude of the difference reduced as age increased and was not accompanied by any macroscopic changes or microscopic changes in the adult animals and was therefore considered to be of no toxicological importance.

To sum up, the NOAEL for reproductive performance in the CD rat is 15000 ppm lauric arginate which is equivalent to at least 1073 mg/kg bw/day based on the lowest average intake by adult rats before pairing and up to 2600 mg/kg bw/day for females during lactation. There was a slight reduction in offspring bodyweight gain just before weaning, a delay in vaginal opening of F1 females and reduced spleen weights among F1 and F2 offspring at 15000 ppm at a point when estimated achieved dosage was in excess of 1900 mg/kg bw/day. However, these effects were transient and were regarded as not toxicologically significant.

Summary of reproduction and developmental toxicity:

Table 16 summarises the NOAEL values established in one generation studies in rats and rabbits and from the results obtained in the two generation study in rats.

Table 16. *Summary of results obtained in studies of one and two generations*

Experiment	**Rats**	**Rabbits**
One generation	Doses: 200, 600 and 2000 mg/kg bw/day. **NOAEL** for dams: 200 mg/kg bw/day. **NOAEL** for foetuses: 2000 mg/kg bw/day	Doses: 100, 300 and 1000 mg/kg bw/day. **NOAEL** for dams: 300 mg/kg bw/day **NOAEL** for foetuses: 1000 mg/kg bw/day.
Two generation	Doses: 2500, 6000 and 15000 ppm. **NOAEL** for reproductive performance and development of F1 and F2: 15000 ppm (equivalent to at least 1073 mg/kg bw/day).	---

Genotoxicity

Several studies were performed to investigate the mutagenicity of lauric arginate, Mirenat-N (a formulation of lauric arginate) and the main metabolite of lauric arginate, N^{α}-lauroyl-L-arginine (LAS). The mutagenicity of lauric arginate, Mirenat-N and LAS was studied at

three different levels. *In vitro* studies were performed to assess if these substances induced gene mutations in bacteria, to determine if lauric arginate and Mirenat-N have gene mutation potential in mammalian cells and if lauric arginate and Mirenat-N produce chromosome aberrations in human lymphocytes. In addition, the mutagenic potential of LAS was evaluated in an *in vivo* micronucleus assay to assess genotoxic hazard.

***Bacterial mutation assay of lauric arginate* (Ames method)** *(Study LMA 038/012403, Huntingdon Life Science, Huntingdon, UK, 2001).*

Introduction:

This study assessed the mutagenicity potential of lauric arginate in a bacterial system (Appendix 2). The bacteria used were four strains of *Salmonella typhimurium* and a mutant strain of *Escherichia coli*. Two independent tests were performed.

This study was conducted following:

- OECD Guidelines for the Testing of Chemicals (1997) Genetic Toxicology: Bacterial Reverse Mutation Test, Guideline 471.
- EEC Annex to Directive 92/69/EEC (1992) Part. B: Methods for Determination of Toxicity, B.13. Other effects – Mutagenicity: *Escherichia coli* – Reverse Mutation Assay. O.J. No. L 383 A, 157.
- EEC Annex to Directive 92/69/EEC. (1992) Part B: Methods for Determination of Toxicity, B.14. Other effects – Mutagenicity: *Salmonella typhimurium* – Reverse Mutation Assay. O.J. No. L 383.A, 160.
- US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5100 Bacterial reverse mutation test. EPA 712-C-98-247.
- JMHW Genotoxicity Testing Guideline, PAB Notification No. 1604 (1 November 1999.)
- ICH (1995 & 1997)
- EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Bacterial strains:

The strains used, *S. typhimurium* TA1535, *S. typhimurium* TA100*, S. typhimurium* TA1537*, S. typhimurium* TA98 and *E. coli* WP2uvrA/pKM101 (CM891) carry additional mutations that render them more sensitive to mutagens. The *S. typhimurium* strains have a defective cell coat, which allows greater permeability of test substances into the cell. All the strains are deficient in normal DNA repair processes. Moreover, 2 of them possess a plasmid pKM101 as does *E. coli*, which introduces an error-prone repair process, resulting in increased sensitivity to some mutagens.

The strains of *S*. *typhimurium* were obtained from the National Collection of Type Cultures, London. The strain of *E. coli* was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.

Each batch of frozen strain was tested for cell membrane permeability, sensitivity to UV light and pKM101 plasmid, which confers resistance to ampicillin. The responses of the strains to a series of diagnostic mutagens were also assessed.

For use in tests, an aliquot of frozen culture was added to nutrient broth and incubated. These cultures provided a known concentration of cells/ml.

Test substance:

LAE Batch 3036 [purity 89.4%[35]] was used as supplied.

Solvent selection and test substance formulation:

The solubility of the test substance was assessed at 50 mg/ml in water and in dimethyl sulphoxide (DMSO). As it was insufficiently soluble in water, the solvent used was DMSO. All concentrations were expressed in terms of the active ingredient of lauric arginate, corrected for purity.

Positive controls:

DMSO was used as the solvent in the presence and absence of S-9 mix. Positive controls were:

- *In the absence of S-9 mix*: Sodium azide, 0.5 μg/plate was used for the TA1535 and TA100 strains, 9-Aminoacridine, 30 μg/plate for the TA1537 strain; 2-Nitrofluorene, 1 μg/plate: for the TA98 strain; and 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2), 0.05 μg/plate for the WP2uvrA/pKM101 (CM891) strain.
- *In the presence of S-9 mix*: 2-Aminoanthracene, 2 μg/plate was used for the TA1535 strain and 10 μg/plate for the WP2uvrA/pKM101 (CM891) strain; and Benzo[a]pyrene, 5 μg/plate for the TA1537, TA98 and TA100 strains.

Negative control:

The solvent DMSO was used as the control in the presence and absence of S-9 mix.

Preparation of S-9 fraction:

The S-9 fraction was prepared from healthy male rats according to the method described by Ames *et al*[36]. Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intra-peritoneal injection in corn oil at a dosage of 500 mg/kg bw. Each batch of S-9 fraction was tested for sterility and efficacy.

Preparation of S-9 mix:

The S-9 mix contained S-9 fraction, sodium phosphate buffer, glucose-6-phosphate and NADPH and NADH.

Dose levels:

Various concentrations of lauric arginate were used.

Method:

First test (range-finding):

The test substance was added to cultures of the 5 tester strains at 7 concentrations separated by approximately half log intervals. The highest concentration tested was 50 mg/ml, which provided a final concentration of 5000 μg/plate, the maximum concentration recommended in the guidelines for this assay. The lowest concentration was 5 μg/plate.

Ten hour bacterial culture, S-9-mix, phosphate buffer, test substance, positive or negative control and molten agar containing histidine/biotin/tryptophan were mixed thoroughly and overlaid on minimal agar in Petri dishes.

Three Petri dishes were used for each treatment.

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[35] The purity of this batch appears in the report as 93.2% because it is expressed as ethyl-N^{α}-lauroyl-L-arginate $HCl \cdot H_2O$. When the purity is expressed as ethyl-N^{α}-lauroyl-L-arginate HCl it is 89.4%.

[36] Ames, B.N.; Mc Canm, J.; Yamasaki, E. Methods for detecting carcinogens and mutagens with Salmonella, mammalian microsome mutagenicity test. *Mutation Res*., 31, 347. 1975.

All plates were incubated before the background material lawn was examined and revertant colonies counted.

First test (range-finding-repeat):
Since only 2 non-toxic concentrations were obtained in the first test, the test was repeated as described above, using a maximum concentration of 150 μg/plate ranging down to 0.15 μg/plate.

Second test:
As a clear negative response was obtained in test 1, a variation of the previous procedure was used in test 2. The variation used was the pre-incubation assay in which the tubes that contained mixtures of bacteria, buffer or S-9 mix and the test substance in DMSO were incubated at 37ºC for 30 minutes with shaking before the addition of the agar overlay. In this test the maximum concentration was 150 μg/plate and a total of 5 concentrations were used down to 1.5 μg/plate.

Stability and formulation analysis:
The stability of the test substance, the test substance in solvent and the achieved concentrations were not determined in this part of the study.

Results:

The absence of microbial contamination was confirmed. The cell cultures were viable and there was a high cell density in all of the cultures. The mean revertant colony counts for the solvent controls were considered to be satisfactory and the appropriate positive control chemicals induced substantial increases in revertant colony numbers. The study was therefore valid.

First test (range-finding): no substantial increases in revertant colony numbers with respect to the control counts were observed with any of the tester strains following exposure to lauric arginate at any concentration, in either the presence or absence of S-9 mix.

Toxicity (observed as thinning of the background lawn of non-revertant cells, together with a reduction in revertant colony numbers) was seen at exposures of 50 μg/plate and above in all *Salmonella typhimurium* strains and at 150 μg/plate and above in the *E. coli* strain.

First test (range-finding-repeat): the maximum exposure concentration selected was 150 μg/plate.

When compared to control counts there were no substantial increases in revertant colony numbers with any of the tester strains following the exposure to lauric arginate in either the presence or absence of S-9 mix.

Toxicity was observed in all strains following exposure of 150 μg/plate.

Maximum concentration of 150 μg/plate was selected for the second test.

***Second test*:** when compared with the control counts, no substantial increases in revertant colony number were observed in any of the bacterial strains following exposure to lauric arginate at any concentration in either the presence or the absence of S-9 mix. Toxicity was seen in all strains following exposure to 150 μg/plate.

Conclusion:

Under the test conditions employed, there was no evidence that lauric arginate had any mutagenic activity in this bacterial system.

Bacterial mutation assay of Mirenat -N (Ames method) *(Study LMA 6/951414, Huntingdon Life Science, Huntingdon, UK, 1995).*

Introduction:

Mirenat-N, a formulation of lauric arginate in a solution of propylene glycol, was tested for potential bacterial mutagenic effects (Appendix 5) using the *in vitro* technique of Ames and co-workers[37,38]. This consisted of exposing mutant strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) to various concentrations of Mirenat-N.

This study was conducted in compliance with the following guidelines:

- OECD Guidelines for Testing of Chemicals No. 471: Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay, 26 May 1983.
- EEC Methods for Determination of Toxicity, Annex to Directive 92/69/EEC, (OJ No. L383A, 29.12.92), Part B, Method B.14. Other effects-Mutagenicity: *Salmonella typhimurium*-Reverse Mutation Assay.
- US Environmental Protection Agency, Method: HG-Gene Muta- *S. typhimurium*: The *Salmonella typhimurium* reverse mutation assay, 1984.
- EU, OECD, USA, Japanese and UK GLP Guidelines.

Experimental Procedure:

Bacterial strains:

The bacterial strains of *S. typhimurium* were the same as those used in the previous study (bacterial mutation assay of lauric arginate) (Appendix 2) and the characteristics were the same.

The strains used were:

- *S. typhimurium* TA1535,
- *S. typhimurium* TA100,
- *S. typhimurium* TA1537,
- *S. typhimurium* TA98, obtained from Professor B. N. Ames, University of California, USA.

Batches of strains were stored at –80ºC. Each batch of frozen strain was tested for cell membrane permeability and where applicable for the pKM101 plasmid which confers resistance to ampicillin.

Test substance:

The test substance was Mirenat-N Batch 00000003 [19.5% solution of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl in propylene glycol[39]].

Solvent selection and test formulation:

The solubility of Mirenat-N was assessed at 50 mg/ml in water. After mixing with water a milky solution was obtained. Therefore, water was chosen as the solvent for use.

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[37] Ames, B.N.; Mc Canm, J.; Yamasaki, E. Methods for detecting carcinogens and mutagens with Salmonella, mammalian microsome mutagenicity test. *Mutation Res.*, 31, 347. 1975.

[38] Maron, D.M.; Ames, B.N. *Mutation Research*, 113, 173. 1983.

[39] The purity of this batch appears in the report as 25% of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl by error due to the fact that HLS did not receive the Certificate of Analysis.

Stability and formulation analysis:

The stability of the test substance, the test substance in solvent and the achieved concentrations were not determined in this study.

Positive controls:

DMSO was used as the solvent in the presence and absence of S-9 mix.

Positive controls were:

- *In the absence of S-9 mix*: N-Ethyl-N'-nitro-N-nitrosoguanidine, 5 μg/plate for the TA1535 strain and 3 μg/plate for strain TA100; 9-Aminoacridine, 80 μg/plate for the TA1537 strain; and 2-Nitrofluorene, 1 μg/plate for the TA98 strain.
- *In the presence of S-9 mix*: 2-Aminoanthracene, 2 μg/plate for the TA1535 and TA1537 strains, 0.5 μg/plate for strain TA98 and 1 μg/plate for the TA100 strain.

Negative control:

The solvent, water, was used in the presence and the absence of S-9 mix.

Preparation of S-9 fraction:

The S-9 fraction was prepared using healthy male Sprague-Dawley rats. Mixed function oxidase systems in the liver were stimulated by Aroclor 1254, administered as a single intra-peritoneal injection in Arachis oil at a dosage of 500 mg/kg bw.

Preparation of S-9 mix:

The S-9 mix contained S-9 fraction, $MgCl_2$, KCl, sodium phosphate buffer, glucose-6-phosphate and NADP.

Dose levels:

Depending on the test, various concentrations of Mirenat-N were used.

Method:

Preliminary toxicity test:

Four concentrations of test substance were assessed for toxicity using the four tester strains. The highest concentration was 50 mg/ml of test substance in the chosen solvent, which provided a final concentration of 5000 µg/plate [50 µg/plate is stated by error in the text but not in the table in the report]. Three 10-fold serial dilutions (500, 50 and 5 µg/plate) of the highest concentration were also tested. The chosen solvent, water, was used as the negative control. Aliquots of 10 hour bacterial culture and 0.5 ml S-9 mix or 0.5 ml 0.1 M phosphate buffer (pH 7.4) were placed in glass bottles. The test solution was added, followed immediately by histidine-deficient agar. The mixture was thoroughly shaken and overlaid onto previously prepared petri dishes containing minimal agar. A single petri dish was used for each dose level. Plates were also prepared without the addition of bacteria in order to assess the sterility of the test substance, S-9 mix and phosphate buffer. All plates were incubated for 3 days. After this period the appearance of the background bacterial lawn was examined and revertant colonies counted.

Mutation test procedure:

Mirenat-N was added to cultures of the four tester strains at six concentrations separated by half log intervals: 5000, 1500, 500, 150, 50 and 15 µg/plate. At a later date, the main test was repeated (mutation test 2) using the same procedures and concentrations of 500, 150, 50, 15 and 5 µg/plate.

Results:

Preliminary toxicity test: the revertant colony counts showed that Mirenat-N was toxic at the highest concentration of 5000 μg/plate to the bacterial strains TA98 and

TA100 and towards TA1537 in the presence of S-9 mix only. Toxicity was also seen towards TA98 at 500 μg/plate in the absence of S-9 mix. Therefore, a concentration of 5000 μg Mirenat-N/plate was chosen as the top dose level in mutation test 1.

Mutation test 1: toxicity was observed towards all the strains between doses of 5000 and 500 μg/plate, except at a dosage of 500 μg/plate in the TA1535 strain in the presence of S-9 mix. Therefore, a concentration of 500 μg Mirenat-N/plate was chosen as the top dose level in the second mutation test.

Mutation test 2: toxicity was observed towards all strains at the highest dosage, 500 μg Mirenat-N/plate, except in the TA1535 strain in the presence of S-9 mix.

No substantial increases in revertant colony numbers of any of the tester strains were observed following treatment with Mirenat-N at any dose level, in the presence or absence of S-9 mix, in either mutation test. The concurrent positive control compounds demonstrated the sensitivity of the assay and the metabolising activity of the liver preparations (S-9 mix).

Conclusion:

Mirenat-N showed no evidence of mutagenic activity in this bacterial system.

Mouse lymphoma assay of lauric arginate *(Study LMA 052/042549, Huntingdon Life Science, Huntingdon, UK, 2004)***.**

Introduction:

Lauric arginate was tested for potential mutagenicity in the mouse lymphoma L5178Y cell mutation test (Appendix 4).

This test system detects a wide range of genetic damage in viable cells capable of forming colonies[40]. The methodology is based on established procedures[41,42,43,44,45,46]. The possible toxicity of the test substance was measured in terms of relative suspension growth (RSG) and relative total growth (RTG)[47].

[40] Cole, J.; Harrington-Brock, K.; Moore, M. The mouse lymphoma assay in the wake of ICH4-Where are we now?. *Mutagenesis*, 14, 265-270. 1999.
[41] Clive, D.; Johnson, K.O.; Spector, J-A.F.S; Batson, A.G.; Brown, M.M.M. *Mutation Research*, 59, 61. 1979.
[42] Amacher, D.E.; Paillet, S.C.; Ray, V.A. *Mutation Research*, 64, 391. 1979.
[43] Amacher, D.E.; Paillet, S.C.; Turner, G.N.; Ray, V.A.; Salsburg, D.S. *Mutation Research*, 72, 447. 1980.
[44] Amacher, D.E.; Turner, G.N. *Environmental Mutagenesis*, 2, 254. 1980.
[45] Cole, J.; Arlett, C.F.; Green, M. The fluctuation test as a more sensitive system for determining induced mutation in L5178Y mouse lymphoma cells. *Mutation Research*, 41, 377-386. 1976.
[46] Cole, J.; Arlett, C.; Green, M.; Lowe, J.; Muriel, W. A comparison of the agar cloning and microtitration techniques for assaying cell survival and mutation frequency in L5178Y mouse lymphoma cells. *Mutation Research*, 111, 371-386. 1983.
[47] Moore, M.; Honma, M.; Clements, J.; Awogi, T.; Bolcsfoldi, G.; Cole, J.; Gollapudi, B.; Harrington-Brock, K.; Mitchell, A.; Muster, W.; Myhr.; O'Donovan, M.; Ouldelhkim, M.; San, R.; Shimada, H.; Stankowski, J. Mouse lymphoma thymidine kinase locus gene mutation assay: International workshop on genotoxicity test procedures workgroup report. *Environmental and Molecular Mutagenesis*, 35, 185-190. 2000.

This study was conducted according to the following guidelines:

- OECD Guidelines for testing of Chemicals No. 476: "Genetic toxicology: *In vitro* mammalian cell gene mutation tests", 1997.
- US EPA (1998): Health Effects Tests Guidelines. OPPTS 870.5300 *In vitro* mammalian cell gene mutation test EPA 712-C-98-221.
- Commission Directive 2000/32/EC Annex 4E-B.17. Mutagenicity-*In vitro* mammalian cell gene mutation test. No. L136/65.
- ICH (1996) Guideline S2A: Guidance on Specific Aspects of Regulatory Gentotoxicity Tests for Pharmaceuticals. PAB/PCD Notification No. 444.
- ICH (1998) Guideline S2B: Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. PMSB/ELD Notification No. 554.
- EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Cells:
The cells used in this study were L5178Y mouse lymphoma (3.7.2.c) cells obtained from the MRC Cell Mutation Unit, University of Sussex, Brighton. These cells are heterozygous at the thymidine kinase locus $TK^{+/-}$. Spontaneous thymidine kinase deficient mutants, $TK^{-/-}$ were eliminated from the cultures by a 24 hour incubation in the presence of methotrexate, thymidine, hypoxanthine and glycine two days prior to storage at –196 ºC, in heat-inactivated donor horse serum (HiDHS) containing 10% DMSO. Cultures were used within 10 days of recovery from frozen stock. Cell stocks are periodically checked for freedom from mycoplasma contamination.

Test substance:
LAE Batch 7446 (purity 88.2%) was used as supplied.

Solvent selection and test substance formulation:
Lauric arginate was soluble at approximately 236 mg/ml in dimethyl sulphoxide (DMSO). Solutions at this concentration and at approximately 118 mg/ml, when dosed at 1% in medium, formed precipitate that settled out. Solutions of lauric arginate at approximately 15 - 59 mg/ml formed cloudy/milky suspensions in medium. Solutions of approximately 7 mg/ml and lower did not form visible precipitates in medium. The maximum concentration tested in the preliminary toxicity test was 60 mg/ml (final concentration in medium of 600 μg/ml), the objective being to test up to and beyond the limit of solubility.

Positive controls:
DMSO was used as the solvent in the absence and the presence of S-9 mix and the positive controls were:

- *In the absence of S-9 mix*: methyl methanesulphonate (MMS), 10 μg/ml (3 hour exposure) or 5 μg/ml (24 h exposure).
- *In the presence of S-9 mix*: 3-methylcholanthrene (3 MC), 2.5 μg/ml.

Negative controls:
DMSO, the solvent, was used in the absence and in the presence of S-9 mix.

Preparation of S-9 fraction:
The S-9 fraction was prepared using healthy male Sprague-Dawley rats. Mixed function oxidase systems in the liver were stimulated by Aroclor 1254, administered as a single intra-peritoneal injection in Arachis oil at a dosage of 500 mg/kg bw.

Preparation of S-9 Mix:
S-9 mix contained S-9 fraction, isocitric acid, NADP in R0.

Dose levels:
The final concentration (µg/ml) of lauric arginate to which the L5178Y cells were initially exposed depended on the test. Sample cultures were analysed for determination of the mutant phenotype.

Method:

Preliminary toxicity test:
The objective of the study was to assess cytotoxicity and to select a range of concentrations for use in the main studies.

Cells were exposed to the test substance at concentrations of 0.29–600 µg/ml for 3 hours in the absence and presence of S-9 mix and for 24 hours in the absence of S-9 mix. For both exposures, a cell suspension of known concentration was incubated before adding media or S-9 mix as appropriate. One culture was prepared for each concentration of the test substance for each test condition. Solvent controls were tested in duplicate for each test condition.

Aliquots of test substance dilution (at 100 times the desired final concentration) or solvent were added to each culture prior to incubation for 3 or 24 hours. At the end of the exposure period, the cells were washed once, resuspended, incubated and sampled after 24 and 48 hours to assess growth in suspension. After sampling at 24 hours the cell density was readjusted. The RSG was used to determine the concentrations of test substance for the main test. There was evidence of a steep toxicity curve in the initial preliminary toxicity test, therefore the test was extended for all conditions to achieve a range of dose levels which spanned the toxicity range of approximately 10-100% RSG.

First and second main mutagenicity tests:
Duplicate aliquots of a suspension of cells had R0 or S-9 mix added as appropriate. Quadruplicate cultures were prepared for solvent controls. Aliquots of test substance dilution (at 100 times the desired final concentration, to give 9/10 concentrations between 10 and 50 µg/ml), solvent or positive control were added, then all cultures were incubated, with continuous shaking. At least four serial dilutions of the test substance were tested. Toxicity was measured in terms of RTG.

Following the 3-hour exposure, the cells were washed once, resuspended to a known nominal concentration and incubated for a further 48 hours to allow for expression of mutant phenotype. The cultures were sampled after 24 and 48 hours to assess growth in suspension. After sampling at 24 hours the cell density was readjusted, to retain a known cell count. After 48 hours, cultures were assessed for cloning efficiency (viability) and mutant potential by plating. Cloning efficiency was assessed by plating a known number of cells per well, two plates being prepared per culture. Mutant potential was assessed by plating a known number of cells per well in selective medium, four plates being prepared per culture. The plates were incubated.

After at least 7 days for viability plates and approximately 10-14 days for mutant plates, the number of empty wells was assessed for each 96-well plate (P0). P0 was used to calculate the cloning efficiency (CE) and mutant frequency (MF). The colony size distribution in the solvent and positive controls was examined to ensure that there was adequate recovery of small colony mutants.

The maximum concentration tested in the first main test was 50 µg/ml, the formulations being added at 1% final volume in medium. The maximum concentrations tested in the second main test in the absence and presence of S-9 mix were 34 and 46 µg/ml respectively, the formulations being added at 1% final volume in medium.

Third and fourth main mutagenicity tests:
Duplicate cultures were treated for 24 hours with test substance, solvent or positive control. Thereafter, the procedure was the same as in the 3-hour treatment. The maximum concentrations in the third and fourth main tests were 45 and 50 µg/ml respectively. A total of 10 and 8 concentrations respectively down to 1 µg/ml were used.

Stability and formulation analysis:
The stability of lauric arginate, the stability and homogeneity of lauric arginate in the vehicle and analysis of achieved concentration were not determined as part of this study.

Results:

***Preliminary toxicity test*:** a 3-hour exposure to lauric arginate at 0.29-600 µg/ml in the absence and the presence of S-9 mix resulted in RSG of 105-0% and 114-0% respectively. A continuous exposure to 0.29-600 µg/ml for 24 hours in the absence of S-9 mix resulted in RSG of 98-0%.

Concentrations used in the main test were based upon these data.

***Test 1 in the absence of S-9 mix*:** a 3-hour exposure to lauric arginate at concentrations of 10-50 µg/ml resulted in RSG values of 96-0%. Cultures exposed to lauric arginate at concentrations of 10-30 µg/ml were assessed to determine mutant frequency. RTG values of 90-29% and cloning efficiencies of 118-93%, relative to the solvent control, were obtained. There were no statistically significant increases in mutant frequency after exposure to concentrations of lauric arginate at acceptable levels of toxicity.

***Test 1 in the presence of S-9 mix*:** a 3-hour exposure to lauric arginate at concentrations of 10-50 µg/ml resulted in RSG values of 101-0%. Cultures exposed to lauric arginate at concentrations of 10-46 µg/ml were assessed for determination of mutant frequency. RTG values of 101-5% and cloning efficiencies of 116-87%, relative to the solvent control, were obtained. There were no statistically significant increases in mutant frequency after exposure to concentration of lauric arginate at acceptable levels of toxicity.

***Test 2 in the absence of S-9 mix*:** a 3-hour exposure to lauric arginate at concentrations of 10-34 µg/ml resulted in RSG values of 89-16%. Cultures exposed to lauric arginate at concentrations of 10-34 µg/ml were assessed for determination of mutant frequency. RTG values of 79-20%, and cloning efficiencies of 120-81%, relative to the solvent control, were obtained. There were no statistically significant increases in mutant frequency after exposure to concentration of lauric arginate at acceptable levels of toxicity.

***Test 2 in the presence of S-9 mix*:** a 3-hour exposure to lauric arginate at concentration of 15-46 µg/ml resulted in RSG values of 93-33%. Cultures exposed to lauric arginate at concentrations of 15-46 µg/ml were assessed for determination of mutant frequency. RTG values of 94-32% and cloning efficiencies of 114-92%, relative to the solvent controls, were obtained. Data presented for concentrations tested span the toxicity range of approximately 30-100% RTG. There were no statistically significant increases in mutant frequency after exposure to concentrations of lauric arginate at acceptable levels of toxicity.

***Test 3 in the absence of S-9 mix*:** a 24-hour exposure to lauric arginate at concentrations of 1-45 µg/ml resulted in RSG values of 110-34%. Cultures exposed to lauric arginate at concentrations of 1-45 µg/ml were assessed for determination of mutant frequency. RTG values of 142-32% and cloning efficiencies of 129-83%,

relative to the solvent control, were obtained. There were no statistically significant increases in mutant frequency after exposure to concentrations of lauric arginate at acceptable levels of toxicity.

***Test 4 in the absence of S-9 mix*:** a 24-hour exposure to lauric arginate at concentration of 1-50 μg/ml resulted in RSG values of 107-20%. Cultures exposed to lauric arginate at concentrations of 1-50 μg/ml were assessed for determination of mutant frequency. RTG values from 115-22% and cloning efficiencies of 130-92%, relative to the solvent control, were obtained. There were no statistically significant increases in mutant frequency after exposure to concentrations of lauric arginate, at acceptable levels of toxicity.

All the tests were regarded as valid as the responses of the various negative and positive controls were within the expected ranges. In particular the positive controls induced large increases in the number of mutant colonies.

Conclusion:

Lauric arginate did not demonstrate mutagenic potential in this *in vitro* cell mutation assay under the experimental conditions described.

Mammalian cell mutation assay of Mirenat-N *(Study LMA 7/951679, Huntingdon Life Science, Huntingdon, UK, 1995)***.**

Introduction:

Mirenat-N, a formulation of lauric arginate in a solution of propylene glycol, was tested for mutagenic potential in the mouse lymphoma L5178Y cell (Appendix 6). The experimental methods employed were based on established procedures[48,49,50,51].

This study was conducted according to the following guidelines:

- OECD Guidelines for Testing of Chemicals No.476: "Genetic toxicology: *In vitro* mammalian cell gene mutation tests". Adopted 4 April, 1984.
- US Environmental Protection Agency, Method: HG-Gene Muta-Somatic Cells: Detection of gene mutations in somatic cells in culture, 1983.
- EEC Directive 87/302/EEC.
- EU, OECD and UK GLP Guidelines.

Experimental Procedure:

Cells:

The cells used were L5178Y mouse lymphoma cells (3.7.2.c) obtained from Dr. J. Cole, Sussex University. These cells are heterozygous at the thymidine kinase locus, $TK^{+/-}$. Spontaneous thymidine kinase deficient mutants, $TK^{-/-}$, were eliminated from the cultures by 24 hours of incubation in the presence of methotrexate, thymidine, hypoxanthine and glycine two days prior to storage at –196ºC. The cells were stored in polypropylene ampoules in heat-inactivated donor horse serum (HiDHS) containing 10% DMSO and used within 7 days of recovery from frozen stock.

[48] Clive, D.; Johnson, K.O.; Spector, J-A.F.S; Batson, A.G.; Brown, M.M.M. *Mutation Research*, 59, 61. 1979.
[49] Amacher, D.E.; Paillet, S.C.; Ray, V.A. *Mutation Research*, 64, 391. 1979.
[50] Amacher, D.E.; Paillet, S.C.; Turner, G.N.; Ray, V.A.; Salsburg, D.S. *Mutation Research*, 72, 447. 1980.
[51] Amacher, D.E.; Turner, G.N. *Environmental Mutagenesis*, 2, 254. 1980.

Media:
The media used were: RPMI 1640, R0p and R10p. Selective medium consisted of cloning medium containing 4 μg/ml trifluorothymidine (TFT).

Test substance:
The test substance was Mirenat-N Batch 00000003 [19.5% solution of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl in propylene glycol[52]].

Solvent selection and test substance formulation:
The maximum solubility of Mirenat-N was 500 mg/ml but when it was dosed at 1% in treatment medium, a heavy precipitate was observed. A maximum final concentration of 2000 μg/ml, at which precipitate was still observed, was used as the upper dose limit in the preliminary toxicity test.

Mirenat-N was diluted in water on the morning of the day of the test. The final concentration of water in the cultures was 1% v/v.

Stability and formulation analysis:
The stability of the test substance, the test substance in solvent and the achieved concentrations were not determined in this study.

Positive controls:
Water or DMSO was the solvent used in the absence and the presence respectively of S-9 mix.

In the absence of S-9 mix ethyl methanesulphonate (EMS), 500 μg/ml was used while, in the presence of S-9 mix 20-methylcholanthrene (20 MC), 2.5 μg/ml was used.

Negative control:
Water, the solvent, was used in the absence and in the presence of S-9 mix.

Preparation of S-9 fraction:
The S-9 fraction was prepared from a group of healthy Sprague-Dawley male rats. Mixed function oxidase systems in the rat liver were stimulated by Aroclor 1254, administered as a single intraperitoneal injection in Arachis oil at a dosage of 500 mg/kg bw.

Preparation of S-9 mix
S-9 mix contained S-9 fraction, isocitric acid and NADP in R0.

Dose levels:
The final concentrations of Mirenat-N used depended on the test.

Preliminary toxicity test:
A cell suspension of known population density was mixed thoroughly and one culture with and without S-9 mix was prepared for each of the 9 concentrations between 15 and 2000 μg/ml of test substance used. A known volume of test substance or solvent was then added to each culture and the cultures mixed for 3 hours. The cells were then washed once with basic medium before mixing for 48 hours. The suspension growth was monitored and adjusted as necessary. The growth of the suspensions of treated cultures, relative to that of the untreated controls, was used as a measure of

[52] The purity of this batch appears in the report as 25% of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl by error due to the fact that HLS did not receive the Certificate of Analysis.

test substance toxicity and a maximum concentration, which reduced cell growth by approximately 80%, selected for the main test.

Main test:
In 2 independent tests, cell suspensions were prepared as above. A known volume of test substance, solvent or positive control was added to each culture. At least 4 serial dilutions were used at 8 concentrations from 100 - 300 µg/ml in the absence of S9 and 100 – 500 µg/ml in the presence of S9 expected to span the LC_{80}-LC_0 range. The procedures followed were those described above. After the 48 hour expression period the cells were assessed for viability and mutant frequency by plating triplicate samples in semi-solid agar. The plates were incubated for 11-12 days before the colonies of L5178Y cells on the plates were counted. The results reported were based on colonies with a diameter of >100 µm.

Results:

***Preliminary toxicity test*:** dose levels of 15-2000 µg/ml in the absence and presence of S-9 mix, resulted in relative growth in suspension (RSG) of 120-1% and 109-0% respectively compared to the solvent control experiments. Concentrations used in the main test were based on these results.

***Main test in the absence of S-9, Test-1 and Test-2*:** the treatment of cells with 100-300 µg/ml in both tests resulted in mean cell growths in suspension of 96-21% and 94-1% respectively. In Test 1, cultures treated with 100, 200, 280 and 300 µg of Mirenat-N/ml and cultures treated with 150, 220, 240 and 280 µg of Mirenat-N/ml in Test 2 were cloned in soft agar and the levels of viability and induced mutation measured. The resulting RTGs were 86 – 24% in Test 1 and 77 – 35% in Test 2, relative to the controls.

No significant increases in mutant frequency were observed after treatment with Mirenat-N in either test.

***Main test in the presence of S-9, Test-1 and Test-2*:** treatment of cells with 100-500 µg/ml in both tests resulted in mean cell growths in suspension of 82-2% and 90-2% respectively. Cultures treated with 200, 300, 400, 425 and 450 µg Mirenat-N/ml in Test 1 and 200, 300, 400 and 450 µg Mirenat-N/ml in Test 2 were cloned in soft agar and the levels of viability and induced mutation measured. The resulting RTGs were 70 – 8% in Test 1 and 83 – 32% in Test 2, relative to the controls.

No significant increases in mutant frequency were observed after treatment with Mirenat-N in either test. The positive control induced highly significant increases in mutant frequency in both tests.

Conclusion:

Mirenat-N did not demonstrate mutagenic potential in this *in vitro* gene mutation assay.

***In vitro mammalian chromosome aberration test of lauric arginate using human lymphocytes** (Study, LMA 039/012517, Huntingdon Life Science, Huntingdon, UK, 2001).*

Introduction:

The ability of lauric arginate to cause chromosomal aberrations in human lymphocytes cultured *in vitro* was assessed in this study (Appendix 3). The culture procedure followed established methodology[53,54,55].

[53] Evans, .J.; O'Riordan, M.L. *Mutation Research*, 31, 135. 1975.

Blood samples were taken from healthy non-smoking male human donors. The blood was pooled and diluted with the tissue culture medium. The cultures were incubated in the presence of phytohaemagglutinin (PHA), which resulted in a high mitotic yield[56]. Following treatment with lauric arginate, cells were arrested at metaphase using the mitotic inhibitor, Colcemid®, so that the chromosomes could be examined for aberrations.

This study was performed as two independent tests. In the first, a 3 hour treatment was used in the presence and in the absence of S-9 mix. The second test consisted of continuous treatment without S-9 mix, and 3 hour treatment with S-9 mix.

This study was conducted according to the following guidelines:

- OECD Guideline for the Testing of Chemicals. (1997) Genetic Toxicology: *in vitro* Mammalian Chromosome Aberration Test, Guideline 473.
- US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5375 *in vitro* Mammalian Chromosome Aberration Test. EPA 712-C-98-223.
- UK GLP Guidelines.

Experimental procedure:

Lymphocyte cultures:
Human blood was collected aseptically from healthy, non-smoking male donors, pooled and diluted with RPMI 1640 tissue culture medium supplemented with foetal calf serum, 1 unit/ml Heparin, 20 I.U./ml penicillin/20 μg/ml streptomycin and 2.0 mM glutamine. Aliquots (blood, medium, phytohaemagglutinin) of the cell suspension were placed in sterile universal containers and incubated for approximately 48 hours. The cultures were gently shaken daily to resuspend the cells.

Test substance:
LAE Batch 3036 [purity 89.4%[57]] was used as supplied.

Solvent selection and test formulation:
Lauric arginate was soluble in DMSO at approximately 400 mg/ml. On dosing at 1% v/v into aqueous tissue culture medium a precipitate was observed at concentrations of 600 μg/ml and above.

Positive controls:
Water was used as the solvent in the presence and in the absence of S-9 mix.

In the absence of S-9 mix, Mitomycin C, 0.1 μg/ml was used while in the presence of S-9 mix, Cyclophosphamide, 6 μg/ml was used.

Negative control:
The solvent, DMSO, was used in the presence and in the absence of S-9 mix.

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[54] Scott, D.; Dean, B.; Danford, N.D.; Kirkland, D.J. Metaphase chromosome aberration assays in vitro in Kirkland, D.J. (Ed.). Basic Mutagenic Test: UKEMS Recommended Procedures. Report of the UKEMS sub-committee on guidelines for mutagenicity testing. Report, Part I revised p. 62. Cambridge University Press, Cambridge. 1990.
[55] Nowell, P.C. *Cancer Research*, 20, 462, 1960.
[56] Nowell, P.C. *Cancer Research*, 20, 462, 1960.
[57] The purity of this batch appears in the report as 93.2% because it is expressed as ethyl-N^{α}-lauroyl-L-arginate HCl·H_2O. When the purity is expressed as ethyl-N^{α}-lauroyl-L-arginate HCl it is 89.4%.

Preparation of S-9 fraction:
The S-9 fraction was prepared from a group of healthy male Sprague-Dawley rats. Mixed function oxidase systems in the rat liver were stimulated by Aroclor 1254, administered as a single intraperitoneal injection in corn oil at a dosage of 500 mg/kg bw.

Preparation of S-9 mix:
S-9 mix contained S-9 fraction, $MgCl_2$, KCl, sodium phosphate buffer pH 7.4, glucose 6 phosphate and NADP.

Dose levels:
The different concentrations of lauric arginate employed depended on the test.

First test
Treatment of cells with test substance: Lauric arginate was added to one set of duplicate cultures to give a total of 8 concentrations from 12.5 to 1600 µg/ml. DMSO, the solvent control, was added to two cultures. Mitomycin C was added to duplicate cultures.

Immediately before treatment of the second set of cultures, medium was removed from each culture and replaced with S-9 mix and the various dilutions of lauric arginate, giving the same series of final concentrations as above. DMSO was added to 2 cultures. Cyclophosphamide was added to duplicate cultures. Three hours after dosing, the cultures were centrifuged. The cell pellets were rinsed and resuspended in fresh medium. They were then incubated for a further 17 hours.

Harvesting and fixation: Two hours before the cells were harvested, mitotic activity was arrested by addition of Colcemid® to each culture and after 2 hours incubation, each cell suspension was centrifuged. The cell pellets were treated with hypotonic solution. After hypotonic incubation freshly prepared cold fixative (3 parts methanol: 1 part glacial acetic acid) was gently added. The cells were then centrifuged. The supernatant was discarded and replaced with fresh fixative. Further fixative replacement was repeated until it became colourless.

Slide preparation: The pellets were resuspended, centrifuged and finally resuspended in fresh fixative. A few drops of the cell suspensions were dropped onto pre-cleaned microscope slides, which were allowed to air-dry. The slides were then stained in 10% Giemsa. After rinsing in buffered water the slides were left to air-dry and then mounted in DPX.

Microscopic examination: The prepared slides were examined by light microscopy and the proportion of mitotic cells per 1000 cells in each culture was recorded except for positive control treated cultures.

From these results, the dose level causing a decrease in mitotic index of 70% of the solvent control value was used as the highest dose level for the metaphase analysis. The intermediate and low dose levels were also selected.

One hundred metaphase figures were examined from each culture. Chromosome aberrations were scored according to the classification of the ISCN (1985). Only cells with 44 - 48 chromosomes were analysed. Polyploid and endoreduplicated cells were noted when seen. The incidence of polyploid metaphase cells, out of 500 metaphase cells, where possible, was determined quantitatively for negative control cultures and all cultures treated with the test substance used in the analysis for chromosomal

aberrations. The number of aberrant metaphase cells in each treatment group was compared with the solvent control value using Fisher's test[58].

Second test
Cultures were initiated and maintained as previously described. In this test a continuous treatment was used in the absence of S-9 mix. In the presence of S-9 mix, a 3 hour treatment was used, as in the first test. The harvest time was at 20 hours for both parts of the test. Duplicate cultures were used for each treatment at 8 concentrations from 12.5 to 300 µg/ml in the absence of S9 mix and 6 concentrations from 25 to 300 µg/ml in the presence of S9 mix and 2 cultures were treated with the solvent control. Positive control cultures were treated as in the first test. Three hours after dosing, the cultures containing S-9 mix were centrifuged and the cell pellets resuspended in fresh medium. They were then incubated for a further 17 hours. Cultures treated in the absence of S-9 mix were incubated for 20 hours. All cultures were treated with Colcemid® 2 hours before the end of the incubation period. They were then harvested, fixed and the slides prepared and examined microscopically.

Stability and formulation analysis:
The stability of the test substance, the test substance in the solvent and analysis of achieved concentration were not determined as part of this study.

Results:

First test:
Toxicity data:
In the absence of S-9 mix, lauric arginate caused a reduction on the mitotic index (MI) to 32% of the solvent control value at 200 µg/ml. In the presence of S-9 mix, lauric arginate caused a reduction of the MI to 31% of the solvent control value at 200 µg/ml. In both cases, the dose levels selected for the metaphase analysis were 50, 100 and 200 µg/ml.

The quantitative analysis for polyploidy showed that lauric arginate caused small, statistically significant, increases ($P<0.01$) in the proportion of polyploid cells at the highest dose level, 200 µg/ml, in both the presence and the absence of S-9 mix.

Metaphase analysis:
In the absence and presence of S-9 mix, lauric arginate caused no statistically significant increases in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Second test:
Toxicity data:
In the absence and in the presence of S-9 mix, lauric arginate at 150 µg/ml caused a reduction in the mitotic index (MI) of 32% and 57% of the solvent control respectively. Consequently, the dose levels selected for the metaphase analysis were 50, 100 and 150 µg/ml.

The quantitative analysis for polyploidy showed that lauric arginate caused small, statistically significant, increases ($P<0.01$) in the proportion of polyploid cells at the highest and intermediate dose level, 150 µg/ml and 100 µg/ml, only in the presence of S-9 mix.

Metaphase analysis:

[58] Fisher, R.A. The exact treatment of 2x2 table in statistical methods for research workers. Hafner Publishing Company, N.Y., USA. 1973.

In the absence and the presence of S-9 mix, lauric arginate caused no statistically significant increases in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

In both tests, the increases in polyploidy were seen mainly at cytotoxic dose levels. It was possible that the increases were related to toxicity of the substance and were not of biological significance[59].

The positive controls, mitomycin C and cyclophosphamide caused large, statistically significant, increases in the proportion of aberrant cells, demonstrating the efficacy of the S-9 mix and the sensitivity of the test system.

Conclusion:

There was no evidence that lauric arginate had clastogenic activity in this *in vitro* cytogenetic test system, under the experimental conditions described. Some evidence of polyploidy-inducing activity, mainly at cytotoxic dose levels, was seen, and is unlikely to be of biological significance.

Metaphase chromosome analysis of human lymphocytes cultured in vitro with Mirenat-N *(Study LMA 8/951852, Huntingdon Life Science, Huntingdon, UK, 1995)**.***

Introduction:

This study evaluated the ability of Mirenat-N, a formulation of lauric arginate in propylene glycol, to cause chromosomal aberrations in human lymphocytes cultured *in vitro* (Appendix 7).

Blood samples were taken from healthy non-smoking male humans donors, pooled, and diluted with tissue culture medium. Subsequently, the cultures were incubated in the presence of phytohaemagglutinin (PHA). Following treatment with Mirenat-N, cells were arrested at metaphase using the mitotic inhibitor, colchicine. The chromosomes, in these metaphase cells, were examined for chromosome aberrations.

The aberrations observed were scored according to the classification of ISCN[60]. The total number of cells containing aberrations both with and without gaps was calculated.

This study was conducted according to the following guidelines:

- OECD Guidelines for Testing of Chemicals No. 473 "Genetic Toxicology: *In vitro* Mammalian Cytogenetic Test". Adopted: 26 May 1983.
- Department of Health Report on Health and Social Subjects No. 35. Guidelines for the testing of chemicals for mutagenicity (1989).
- EEC Methods for Determination of Toxicity, Annex to Directive 92/69/EEC (OJ No. L383A, 29.12.92) Part B, Method B. 10. Other effects – Mutagenicity. *In vitro* Mammalian Cytogenetic Test. Incorporating modifications detailed in the Guidance Note of Competent Authorities for the implementation of Directive 92/32/EEC.
- EU, OECD and UK GLP Guidelines.

[59] Muehlbauer, P.A.; Dobo, K.L.; Guzzie, P.J. *Environmental and Molecular Mutagenesis*, 33, 46. 1999.
[60] Harnden, D.G.; Klinger, H.P. ISCN: An International System for Human Cytogenetic Nomenclature. Eds. S. Karger; A.G, Basel. 1985.

Experimental procedure:

Lymphocyte cultures:
Human blood was collected aseptically from healthy, non-smoking male donors, pooled and diluted with tissue culture medium. Lymphocytes were separated by centrifugation. After repeated washing and centrifugation the cells were suspended in culture medium, foetal calf serum and a known concentration of phytohaemagglutinin. The cell suspension was then placed in wells of multiwell tissue culture plates and incubated for approximately 48 hours.

Test substance:
Mirenat N Batch 00000003 [19.5% solution of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl in propylene glycol] was used.

Solvent and dose selection:
Mirenat-N proved to be soluble in water at a concentration of 500 mg/ml. However, when this was dosed into tissue culture medium at 1% v/v, it formed a heavy precipitate. The highest concentration used was 2000 μg/ml, as this concentration produced a slight precipitate.

Positive controls:
DMSO and water was the solvent used in the absence and in the presence respectively of S-9 mix.

In the absence of S-9 mix ethyl methanesulphonate, 250, 500 and 750 μg/ml was used while in the presence of S-9 mix cyclophosphamide, 2.5, 5, 10 and 15 μg/ml was used.

Negative controls:
Water was used as the negative control.

Preparation of S-9 fraction:
The S-9 fraction was prepared from a group of healthy male Sprague-Dawley rats. Mixed function oxidase systems in the rat liver were stimulated by Aroclor 1254, administered as a single intraperitoneal injection in Arachis oil at a dosage of 500 mg/kg bw.

Preparation of S-9 mix:
S-9 mix contained S-9 fraction, $MgCl_2$, KCl, sodium orthophosphate buffer pH 7.4, glucose 6 phosphate and NADP.

Dose levels:
The concentrations of Mirenat-N employed depended on the test.

First test:
Treatment of cells with test substance: Mirenat-N was added to a set of duplicate cultures to give 8 concentrations from 15.6 to 2000 μg/ml. Water, the solvent control, was added to four cultures. Ethyl methanesulphonate, was added to duplicate cultures. S-9 mix was added to each culture in a second set of duplicate cultures, followed by various dilutions of Mirenat-N, giving the same final concentrations as above. Water was added to four cultures. Cyclophosphamide was added to duplicate cultures. Three hours after dosing, the cultures containing the S-9 mix were centrifuged and the cell pellets resuspended in fresh medium. They were then incubated for a further 15 hours. The cultures treated in the absence of S-9 mix were incubated for 18 hours.

Harvesting and fixation: Two hours before the cells were harvested, mitotic activity was arrested by addition of colchicine to each culture at a known final

concentration. After 2 hours incubation, each cell suspension was centrifuged. The cell pellets were treated with a hypotonic solution. After hypotonic incubation, the suspensions were centrifuged and the cell pellets fixed for at least two hours.

Slide preparation: The pellets were aspirated, centrifuged and finally resuspended in fresh fixative. The cell suspensions were dropped onto pre-cleaned microscope slides, and allowed to air-dry. The slides were then placed in buffered distilled water, stained in Giemsa, rinsed in buffered distilled water, left to air-dry and then mounted.

Microscopic examination: The slides were examined by light microscopy. The proportion of mitotic cells per 1000 cells in each culture was recorded except for positive control treated cultures. From these results the dose level causing a decrease in mitotic index of approximately 50% of the solvent control value was used as the highest dose level for the metaphase analysis. The intermediate and low doses were also selected. The concentration of each positive control compound selected for analysis was the lowest concentration dosed unless a preliminary scan of metaphase figures indicated an insufficient level of aberrant cells. One hundred metaphase figures were examined from each culture. Only cells with 44 - 46 chromosomes were analysed. The number of aberrant metaphase figures in each treatment group was compared with the solvent control value using Fisher's test[61].

Second test:
Cultures were initiated and maintained as previously described. Cultures to be harvested 32 hours after initiation of treatment were treated with:

- 5 concentrations from 125 to 2000 µg/ml Mirenat-N in the absence of and
- 3 concentrations from 250 to 1000 µg/ml in the presence of S-9 mix.

Seven concentrations were used for the second 18 hours test:

- 125 to 2000 of Mirenat-N in the absence of S-9 mix, and
- 125 to 1000 µg/ml of Mirenat-N in the presence of S-9 mix.

Duplicate cultures were used for each treatment.

Four cultures were treated with the solvent control.

For the 32 hour harvest, ethyl methanesulphonate and cyclophosphamide were added to duplicate cultures. Three hours after dosing, the cultures containing S-9 mix were centrifuged and the cell pellets resuspended in fresh medium. They were then incubated for a further 15 or 29 hours. Cultures treated in the absence of S-9 mix were incubated for 18 or 32 hours. All cultures were treated with colchicine 2 hours before the end of the incubation period. They were then harvested, fixed and the slides prepared as previously described. The slides were then examined microscopically. For the 32 hour harvest only 1 dose level was selected for analysis. This was the same as the highest dose level chosen from the 18 hour harvest.

Stability and formulation analysis:
The stability of the test substance, the test substance in the solvent and analysis of achieved concentration were not determined as part of this study.

Results:

First test:
Toxicity data:

[61] Fisher, R.A. The exact treatment of 2x2 table in statistical methods for research workers. Hafner Publishing Company, N.Y., USA. 1973.

In the absence of S-9 mix, Mirenat-N reduced the MI to 40% of the solvent control at the highest dose concentration, 2000 μg/ml. A concentration of 500 μg/ml proved to be the highest analysable. The concentrations chosen for metaphase analysis were 125, 250 and 500 μg/ml.

In the presence of S-9 mix, the MI was reduced to 11% of the solvent control at 2000 μg/ml of Mirenat-N. The 500 μg/ml concentration was the highest dose level selected for analysis as higher concentrations were highly toxic. The lower concentrations selected for the metaphase analysis were 125 and 250 μg/ml.

The concentrations of the positive control compounds selected for analysis were ethyl methanesulphonate at 500 μg/ml and cyclophosphamide at 15 μg/ml.

Metaphase analysis:
Mirenat-N caused no statistically significant increases in the proportion of metaphase figures with chromosomal aberrations.

Second test, 18 hours harvest:
Toxicity data:
Mirenat-N, in the absence of S-9 mix, reduced the MI to 39% of the solvent control. The highest analysable concentration was 500 μg/ml giving a relative MI index of 75%. Thus, the lower concentrations selected were 250 and 125 μg/ml.

In the presence of S-9 mix, Mirenat-N gave a mitotic index of 61% of the solvent control value at the highest concentration, 1000 μg/ml. This was selected for analysis with the lower concentrations of 800 and 700 μg/ml.

Metaphase analysis:
Mirenat-N caused no statistically significant increases in the proportion of aberrant cells in either the absence or presence of S-9 mix.

Second test, 32 hours harvest:
Toxicity data:
In the absence of S-9 mix, Mirenat-N at a concentration of 2000 μg/ml reduced the mitotic index to 6% of the solvent control value. Consequently, the highest concentration selected for analysis was 500 μg/ml, which reduced the mitotic index to 66% of the solvent control.

In the presence of S-9 mix, Mirenat-N was relatively non-toxic (MI 98%) to the cells at the highest concentration, 1000 μg/ml. As this was the highest concentration analysed at 18 hours it was selected for analysis.

Metaphase analysis:
Mirenat-N did not cause any statistically significant increase in the proportion of aberrant cells in either the absence or presence of S-9 mix.

In both tests the positive control compounds, ethyl methanesulphonate and cyclophosphamide, caused large, statistically significant, increases in the proportion of aberrant cells. This demonstrated the efficacy of the S-9 mix and the sensitivity of the test system.

Conclusion:
Mirenat-N did not show evidence of clastogenic activity in this *in vitro* cytogenetic test system.

Bacterial reverse mutation test of N^{α}-lauroyl-L-arginine *(Study CD02/8400T, Centro de investigación y desarrollo aplicado, S.A.L., CIDASAL, Spain, 2003)***.**

Introduction:

This study assessed the possible mutagenic potential of the main metabolite of lauric arginate, N^{α}-lauroyl-L-arginine (LAS), (Appendix 8) using four strains of *Salmonella typhimurium* and one strain of *Escherichia coli* in the presence and the absence of S-9 (the metabolic activation system).

This study was performed according to the following guideline:

- OECD Guidelines for Testing of Chemicals No. 471: Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay, updated 21 July 1997.
- EU, OECD and Spanish GLP Guidelines.

Experimental Procedure:

Bacterial strains:
The following strains were used:

- *S. typhimurium* TA1535 CECT 882,
- *S. typhimurium* TA1537 CECT 883,
- *S. typhimurium* TA98 CECT 880,
- *S. typhimurium* TA100 CECT 881, and
- *E. coli* WP2 uvrA pKM101 NCIMB 11703.

All strains were supplied by Colección Española de cultivos tipo (University of Valencia, Spain) except *E. coli*, which was supplied by NCIMB Ltd., Scotland.

Before initiating the study, the strains were grown on Petri plates with nutrient agar to obtain pure cultures. The histidine and tryptophan requirements were checked.

Test substance:
LAS Batch Q-98.251.6 [purity 98.6%] was used as supplied.

Solvent solubility and test substance formulation:
The solvent, DMSO, was gradually added to 100 mg of LAS. The maximum concentration of LAS possible was 50 mg/ml, which is the recommended maximum in OECD Guideline no. 471.

Stability and formulation analysis:
The stability of the test substance, the test substance in the solvent and analysis of achieved concentration were not determined as part of this study.

Positive control:
With the exception of sodium azide, for which the solvent was water, DMSO was used as the solvent.

In the absence of S-9 mix:
- 2-nitrofluorene, 1 and 10 μg/plate, was used for strain TA98,
- sodium azide, 1 and 10 μg/plate, was used for strains TA100, TA1535,
- 9-aminoacridine, 5 and 50 μg/plate, for strain TA1537 and
- 4-nitroquinoline oxide, 0.5 and 1 μg/plate for strain WP2 uvrA pKM101.

In the presence of S-9 mix:
- 2-aminoanthracene, 1 and 10 μg/plate was used for all strains.

Negative control:
With the exception of sodium azide, which utilised water, DMSO was the negative control.

Preparation of S-9 fraction:
The metabolic activation system (S-9) was supplied by IFFA Credo (France).

Preparation of the S-9 mix:
The S-9 mix consisted of: S-9, $MgCl_2\,6H_2O$-KCl, glucose-6-phosphate, NADP, phosphate buffer and bidistilled water.

Dose levels:
LAS was administered at various concentrations in bacterial culture, phosphate buffer (experiment without S-9 mix) or S-9 mix. The concentrations used for the preliminary study were up to 5000 μg/plate.

Method:
Phosphate buffer or S-9 mix and a dilution of the test substance to give 6 concentrations from 156.25 to 5000 μg/plate were added to the bacterial cultures. For the Controls, the corresponding solvent for each product was added. The mixes were incubated and added to melted top agar, containing biotin and histidine, for *Salmonella typhimurium* and a sterile solution of tryptophan and nutrient broth to top agar for *Escherichia coli*. The contents of the test tubes were mixed rapidly, and poured onto plates containing a base layer of minimal agar, which was allowed to set and the plates incubated.

The experiment was repeated using fresh bacterial cultures.
In parallel to the test substance LAS, N^{α}-lauroyl-L-arginine, the positive controls were tested in order to check the sensitivity of the strains used to mutagenic agents.

Statistical analysis:
A statistical analysis of the test substance concentrations was performed for all bacterial strains, with and without metabolic activation, using 1-way analysis of variance.

Results:
All results obtained were compared with the revertant colony counts of the negative controls (treated only with the solvent).

Experiment 1:
LAS, at a concentration of 5000 μg of LAS/plate was toxic to the TA1535, TA1537 and TA100 strains of *S. typhimurium* without S-9 and to TA1537 with S-9.

In this experiment, no mutagenic response was observed in any of the tested strains, either with or without S-9.

Experiment 2:
Toxicity was observed at 5000 μg LAS/plate in the TA1535 and TA1537 strains of *S. typhimurium* without S-9 and in strain TA1537 with S-9.

No mutagenic response was observed in any of the tested strains, either with or without S-9.

The results obtained with the positive controls showed that all bacterial strains responded positively, and the metabolic activation system functioned as expected in both tests.

Conclusions:

Under the conditions described, LAS produced no mutagenic activity in any of the 5 bacterial strains used.

In vivo micronucleus assay with N^{α}-lauroyl-L-arginine *(Study CD02/8401T, Centro de Investigación y desarrollo Aplicado, S.A.L., CIDASAL, Spain, 2003)***.**

Introduction:

This study evaluated the mutagenic potential of N^{α}-lauroyl-L-arginine (LAS), the main metabolite of lauric arginte, by measuring its effects on the induction of micronuclei due to lagging chromosome fragments (clastogenicity) or whole chromosomes (aneugenicity) in polychromatic erythrocytes (PCE) from mouse bone marrow following a single oral administration 24 or 48 hours earlier (Appendix 9).

This study was performed according to the following guidelines:

- OECD Guidelines for the testing of chemicals, Guideline 474, 21 July 1997.
- Commission Directive 2000/32/EC of 19 May 2000 adapting to technical progress for the 26th time Council Directive 67/548/EEC.
- ICH Topic S2A, Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, September 1995.
- ICH Topic S2B, Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals, July 1997.
- EU, OECD and Spanish GLP Guidelines.

Experimental Procedure:

Two experiments were performed:

- The first one was a preliminary dose-range finding study to select a dose for the main study, and
- The main study.

In both studies LAS was administered once orally by gavage.

Animal management:

Male and female Crl:CD-1® (ICR) IGS mice were obtained from Charles River, France. In the preliminary study an acclimatisation period of 6 days was allowed before starting any experimental work. Animals were housed 5 or 3/cage with food and water available *ad libitum*. Room temperature was 19-25ºC, relative humidity 50 to 70%, and a 12 hour artificial light/dark cycle was maintained in the room. Animals were 42-49 days of age at the start of treatment and within the bodyweight range 27.0-32.0 and 24.0-26.0 g for males and females respectively on Day 1 of treatment.

In the preliminary test, two groups of 8 male and 8 female animals received LAS once by oral gavage, at dosages of 1300 or 1700 mg/kg bw in a dose volume of 10 or 20 ml/kg respectively.

For the main study male animals of the same strain, age and in the weight range 28-32 g were used. The acclimatisation period was 8 days and the animals were housed 5/cage, otherwise the conditions were the same as those described previously. One group of 10 animals received LAS once by oral gavage, at a dosage of 2000 mg/kg bw in a dose volume of 20 ml/kg. A second group of 10 animals received the vehicle, 0.5% carboxymethyl cellulose + Tween 80 once at the dosage volume and acted as a negative control. A third group of 5 animals were given 50 mg/kg bw of

cyclophosphamide in bi-distilled water once at the same dosage volume and acted as a positive control.

Test substance:
LAS Batch Q-98.251.6 [purity 98.6%] was used as supplied.

Test substance formulation:
LAS was administered as a suspension in 0.5% carboxymethyl cellulose and 1% Tween 80 in bidistilled water.

Stability and formulation analysis:
The stability of the test substance, the test substance in the formulation and analysis of achieved concentration were not determined as part of this study.

Method preliminary study:
Two animals given LAS at the higher dose were sacrificed 48 hours post-administration. The bone marrow of both femurs was suspended in foetal calf serum and smears prepared. The slides were examined microscopically to assess their suitability.

The test item, LAS (N^{α}-lauroyl-L-arginine), induced no toxicity at the high dose in males or females. In addition, 48 hours after treatment with LAS the analysis of the smears showed no toxic effects on the bone marrow. Therefore, the main study was carried out at a dose level of 2000 mg/kg using only male mice.

Method main study:
Bone marrow extraction: The mice were sacrificed 24 or 48 hours after administration. The bone marrow was removed from both femurs, suspended in foetal calf serum, centrifuged and the supernatant discarded. The pellet was resuspended in foetal calf serum and 3 slides per animal prepared.

Data collection: The slides prepared from each animal were analysed. Two thousand polychromatic erythrocytes (PCE) were counted for each animal and the number of micronucleated polychromatic erythrocytes (MNPCE) and the number of micronucleated normochromatic erythrocytes (NCE) were recorded. For each animal 1000 erythrocytes were counted and the ratio of PCE/(PCE+NCE) was determined in order to assess bone marrow toxicity. The micronuclei were identified according to the criteria established by Schmid[62,63].

Statistical analysis: Statistical analysis was performed on frequency of MNPCE/1000 PCE and the ratios PCE/(PCE+NCE) for each treatment group and sampling time. The results were compared with the negative control. ANOVA and Student's t test were used.

Systemic exposure study: The systemic exposure to the test substance was determined on 2 occasions using 19 male mice of the same strain and body-weight range obtained from the same supplier as those used in the main study. Seven mice chosen from the animals received for the preliminary study and not treated previously were given the vehicle or the test item, LAS (N^{α}-lauroyl-L-arginine), at approximately 1700 mg/kg. The distribution of the animals into groups and the blood sampling times were as indicated below:

[62] Krishna, G.; Hayashi, M. *Mutation Research*, 455, 155-166. 2000
[63] Schmid, W. *Mutation Research*, 31, 9-15. 1975

Treatment	Blood sampling time		
	0.5 hours	1 hour	3 hours
Negative Control	1	---	---
LAS (1700 mg/kg)	2	2	2

Subsequently 12 mice from the same supplier were given the vehicle or the test substance, LAS (N^{α}-lauroyl-L-arginine), at approximately 1700 mg/kg. The body-weight range of the animals on the day of administration was 25-35 g. The distribution into the different groups and the blood sampling times are indicated below:

Treatment	Blood sampling time		
	0.5 hours	1 hour	3 hours
Negative Control	2	2	2
LAS (1700 mg/kg)	2	2	2

Obtaining and storage of samples: Plasma samples were obtained from the treated animals at the designated sampling times on 2 occasions. The samples from each animal were centrifuged at 3000 rpm and the plasma collected. A pool of plasma from all the animals of each group was prepared at each sampling time, giving a total of 10 samples to be analysed. The samples were immediately frozen at –20 ± 5°C until their analysis. The samples were delivered to the Sponsor for analysis.

Determination of LAS levels in plasma: The determination of plasma levels of the product was carried out at Laboratorios Miret, S.A. (LAMIRSA) in November 2002 using High-Performance Liquid Chromatography (HPLC).

Results:

Preliminary study:

No mortality and no observable signs of toxicity were observed in the animals treated with 1300 or 1700 mg/kg bw of LAS. There were no differences in the toxic responses of males and females. Therefore, the main study was carried out in male mice at a dose level of 2000 mg/kg bw.

The bone marrow of the two animals that received the high dose level of LAS showed no toxic effects.

Main study:

No mortality or signs of toxicity were observed in the animals given LAS at a dose level of 2000 mg/kg bw. Similarly animals given the negative or the positive control substances showed no evidence of toxic effects.

There was no significant increase in the frequency of MNPCE/1000 PCE in the bone marrow of the LAS treated mice. Thus, LAS is non-mutagenic under the experimental conditions of this assay. However, in animals given the positive control, cyclophosphamide, at the dose of 50 mg/kg bw, there was a significant increase in the frequency of MNPCE/1000 PCE.

The toxic effects on the bone marrow of treated mice were assessed by comparing the values of the ratio of PCE/(PCE+NCE). No significant reduction in the ratio was observed when comparing the vehicle control and the groups given LAS at either sampling time. Comparing the negative control group with the positive control group, treated with 50 mg/kg bw of cyclophosphamide, no statistically significant reduction

was observed although a slight decrease in the ratio was observed in the group given LAS and in the positive control group.

In the metabolism-toxicokinetic studies with lauric arginate (Appendix 17, 18), LAS was metabolised and transformed into arginine and lauric acid. It was expected that the oral administration of a single dose of LAS to mice would produce similar results with respect to the possible metabolism of the test substance. No LAS was detected in the plasma of the mice examined.

Conclusions:

When LAS was administered by oral gavage at a dose of 2000 mg/kg bw, there was no biologically significant increase in the frequency of MNPCE in the bone marrow of the treated mice at any sampling time. Therefore, LAS was considered to be non-mutagenic.

Summary of the results of mutagenicity tests:

Table 17 is a summary of the results obtained in each experiment reported in this section:

Table 17: *Summary of results obtained in the mutagenicity experiments*

Experiment	Test substance	Strains and Cells	Dose range	Results
Ames test (*in vitro*)	*Lauric arginate*	*Salmonella typhimurium*: TA1535, TA1537, TA100, TA98 *Escherichia coli*: WP2uvrA/pKM101 (CM891)	1.5-150 µg/plate	No mutagenic activity
	Mirenat-N	*Salmonella typhimurium*: TA1535, TA1537, TA100, TA98	5-5000 µg/plate	No mutagenic activity
	LAS	*Salmonella typhimurium*: TA1535, TA1537, TA100, TA98 *Escherichia coli*: WP2uvrA/pKM101 (CM891)	156.3-5000 µg/plate	No mutagenic activity
Mammalian Cell Mutation (*in vitro*)	*Lauric arginate*	L5178Y mouse lymphoma cell	1-50 µg/ml	No mutagenic activity
	Mirenat-N	L5178Y mouse lymphoma cell	100-300 µg/ml	No mutagenic activity
Chromosome Aberration Test (*in vitro*)	*Lauric arginate*	Human lymphocytes	50-200 µg/ml	No clastogenic activity
	Mirenat-N	Human lymphocytes	125-1000 µg/ml	No clastogenic activity
Micronucleus Test (*in vivo*)	*LAS*	*In vivo* experiment performed Mice Crl:CD-1. Analysis of bone marrow	2000 mg/kg bw	No mutagenic activity

SPECIAL STUDIES

Toxicity of metabolites, degradation products and impurities

Acute oral toxicity of N^{α}-lauroyl-L-arginine to the rat *(Study CD02/8399T, Centro de Investigación y Desarrollo Aplicado, S.A.L. (CIDASAL), Sta. Perpètua de Mogoda, Spain, 2003).*

Introduction:

The study assessed the toxicity of N^{α}-lauroyl-L-arginine (LAS) following administration of a single oral dose to the rat (Appendix 56). The data are of interest because metabolism-toxicokinetic studies have indicated that in rats lauric arginate was rapidly metabolised to LAS and then to arginine (Appendix 17, 18, 21).

This study was carried out following the EEC Methods for the determination of toxicity, Annex Directive 92/69/EEC (OJ No. L383A, 29.12.92), Part B, Method B.1. Acute toxicity (oral) and in compliance with the EU, OECD and Spanish GLP Guidelines.

Experimental Procedure:

Animal Management:

Male and female Sprague Dawley CD (Crl:CD® (SD) IGS BR) rats obtained from Charles River Laboratories (Spain) were used. An acclimatisation period of at least 5 days was allowed before starting any experimental work. Animals were housed 5/sex/cage with food and water available *ad libitum* except for approximately 4 hours after dosing. Room temperature was set to achieve 22-25ºC, relative humidity 30 to 65% and a 12 hour artificial light/dark cycle was maintained. Animals were approximately 5 weeks of age and within the bodyweight range 100-121 g on selection before dosing.

A group of 5 male and 5 female animals received a single dose of 2000 mg/kg bw of LAS by oral gavage at a dose volume of 10 ml/kg bw.

Substance:

LAS Batch Q-98.251.6 [purity 98.6%] was used as supplied.

Test substance formulation:

LAS was formulated at a concentration of 20% in 0.5% of carboxymethyl cellulose + 1% Tween 80 in bidistilled water.

Stability and formulation analysis:

The stability of the test substance, the test substance in the selected vehicle and achieved concentrations were not determined in this study.

Observations and terminal investigations:

Frequent observations were made of rats were on the day of test substance administration. Subsequently, they were observed at least twice a day for 14 days. Rats were weighed on days 1 (before dosing), 8 and 15 (before necropsy). At the end of the observation period, all rats were killed and subjected to macroscopic examination. This included examination of the external appearance of superficial tissues and organs followed by the cranial, thoracic and abdominal cavities.

Results:

Clinical signs and mortality:

There were no deaths among the treated animals. No clinical signs were recorded in any of the animals.

Bodyweight gain:
Mean body weight gain of the treated rats was within normal limits.

Macroscopic examination:
No macroscopic changes were observed at necropsy at the end of the study (day 15).

Conclusion:
The median lethal dose (LD_{50}) of LAS following administration of a single oral dose to Sprague Dawley rats was found, in this study, to be higher than 2000 mg/kg bw. Thus, in accordance of the Directive 93/21/EEC and in accordance with the Approved Criteria for Classifying Hazardous Substances [NOHSC:1008(2004)], LAS is not classified as harmful if swallowed.

Bacterial reverse mutation test of N^{α}-lauroyl-L-arginine *(Study CD02/8400T, Centro de investigación y desarrollo aplicado, S.A.L., CIDASAL, Spain, 2003)***.**

Introduction:
This study assessed the possible mutagenic potential of the main metabolite of lauric arginate, N^{α}-lauroyl-L-arginine (LAS), (Appendix 8) using four strains of *Salmonella typhimurium* and one strain of *Escherichia coli* in the presence and the absence of S-9 (the metabolic activation system).

This study was performed according to the following guideline:

- OECD Guidelines for Testing of Chemicals No. 471: Genetic Toxicology: *Salmonella typhimurium*, Reverse Mutation Assay, updated 21 July 1997.
- EU, OECD and Spanish GLP Guidelines.

Experimental Procedure:
Bacterial strains:
The following strains were used:
- *S. typhimurium* TA1535 CECT 882,
- *S. typhimurium* TA1537 CECT 883,
- *S. typhimurium* TA98 CECT 880,
- *S. typhimurium* TA100 CECT 881, and
- *E. coli* WP2 uvrA pKM101 NCIMB 11703.

All strains were supplied by Colección Española de cultivos tipo (University of Valencia, Spain) except *E. coli*, which was supplied by NCIMB Ltd., Scotland.
Before initiating the study, the strains were grown on Petri plates with nutrient agar in order to obtain pure cultures. The histidine and tryptophan requirements were checked.

Test substance:
LAS Batch Q-98.251.6 [purity 98.6%] was used as supplied.

Solvent solubility and test substance formulation:
The solvent, DMSO, was gradually added to 100 mg of LAS. The maximum concentration of LAS possible was 50 mg/ml, which is the recommended maximum in OECD Guideline no. 471.

Stability and formulation analysis:
The stability of the test substance, the test substance in the solvent and analysis of achieved concentration were not determined as part of this study.

Positive control:
Water was the solvent for sodium azide; DMSO was used to dissolve all the others.

In the absence of S-9 mix 2-nitrofluorene, 1 and 10 μg/plate, was used for strain TA98, sodium azide, 1 and 10 μg/plate, was used for strains TA100, TA1535, 9-aminoacridine, 5 and 50 μg/plate, for strain TA1537 and 4-nitroquinoline oxide, 0.5 and 1 μg/plate for strain WP2 uvrA pKM101. In the presence of S-9 mix 2-aminoanthracene, 1 and 10 μg/plate was used for all strains.

Negative control:
With the exception of sodium azide, which utilised water, DMSO was the negative control.

Preparation of S-9 fraction:
The metabolic activation system (S-9) was supplied by IFFA Credo (France).

Preparation of the S-9 mix:
The S-9 mix consisted of: S-9, $MgCl_2$ $6H_2O$-KCl, glucose-6-phosphate, NADP, phosphate buffer and bidistilled water.

Dose levels:
LAS was administered at various concentrations in bacterial culture, phosphate buffer (experiment without S-9 mix) or S-9 mix. The concentrations used for the preliminary study were up to 5000 μg/plate.

Method:
Phosphate buffer or S-9 mix and a dilution of the test substance to give 6 concentrations from 156.25 to 5000 μg/plate were added to bacterial culture. For the Controls, the corresponding solvent for each product was added. The mixes were incubated and added to melted top agar, containing biotin and histidine, for *Salmonella typhimurium* and a sterile solution of tryptophan and nutrient broth to top agar for *Escherichia coli*. The contents of the test tubes were mixed rapidly, and poured onto plates containing a base layer of minimal agar, which was allowed to set and the plates incubated.

The experiment was repeated using fresh bacterial cultures.

In parallel to the test substance LAS, N^{α}-lauroyl-L-arginine, the positive controls were tested in order to check the sensitivity of the strains used to mutagenic agents.

Statistical analysis:
Statistical analysis of the test substance concentrations was performed for all bacterial strains, with and without metabolic activation, using 1-way analysis of variance.

Results:

All results obtained were compared with the revertant colony counts of the negative controls (treated only with the solvent).

Experiment 1:
LAS, at a concentration of 5000 μg of LAS/plate was toxic to the TA1535, TA1537 and TA100 strains of *S. typhimurium* without S-9 and to TA1537 with S-9.

In this experiment, no mutagenic response was observed in any of the tested strains, either with or without S-9.

Experiment 2:
Toxicity was observed at 5000 μg LAS/plate in the TA1535 and TA1537 strains of *S. typhimurium* without S-9 and in strain TA1537 with S-9.

No mutagenic response was observed in any of the tested strains, either with or without S-9.

The results obtained with the positive controls showed that all bacterial strains responded positively, and the metabolic activation system functioned as expected in both tests.

Conclusions:
Under the conditions described, LAS produced no mutagenic activity in any of the 5 bacterial strains used.

In vivo micronucleus assay with N^{α}-lauroyl-L-arginine *(Study CD02/8401T, Centro de Investigación y desarrollo Aplicado, S.A.L., CIDASAL, Spain, 2003)***.**

Introduction:
This study evaluated the mutagenic potential of N^{α}-lauroyl-L-arginine (LAS), the main metabolite of lauric arginte, by measuring its effects on the induction of micronuclei due to lagging chromosome fragments (clastogenicity) or whole chromosomes (aneugenicity) in polychromatic erythrocytes (PCE) from mouse bone marrow following a single oral administration 24 or 48 hours earlier (Appendix 9).

This study was performed according to the following guidelines:

- OECD Guidelines for the testing of chemicals, Guideline 474, 21 July 1997.
- Commission Directive 2000/32/EC of 19 May 2000 adapting to technical progress for the 26th time Council Directive 67/548/EEC.
- ICH Topic S2A, Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, September 1995.
- ICH Topic S2B, Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals, July 1997.
- EU, OECD and Spanish GLP Guidelines.

Experimental Procedure:
Two experiments were performed. The first one was a preliminary dose-range finding study to select a dose for the main study. In both studies LAS was administered once orally by gavage.

Animal management:
Male and female Crl:CD-1® (ICR) IGS mice were obtained from Charles River, France. In the preliminary study an acclimatisation period of 6 days was allowed before starting any experimental work. Animals were housed 5 or 3/cage with food and water available *ad libitum*. Room temperature was 19-25ºC, relative humidity 50 to 70%, and a 12 hour artificial light/dark cycle was maintained in the room. Animals were 42-49 days of age at the start of treatment and within the bodyweight range 27.0-32.0 and 24.0-26.0 g for males and females respectively on Day 1 of treatment.

In the preliminary test, two groups of 8 male and 8 female animals received LAS once by oral gavage, at dosages of 1300 or 1700 mg/kg bw in a dose volume of 10 or 20 ml/kg respectively.

For the main study, male animals of the same strain, age and in the weight range 28-32 g were used. The acclimatisation period was 8 days and the animals were housed 5/cage, otherwise the conditions were the same as those described previously. One group of 10 animals received LAS once by oral gavage, at a dosage of 2000 mg/kg bw in a dose volume of 20 ml/kg. A second group of 10 animals received the vehicle, 0.5% carboxymethyl cellulose + Tween 80 once at the dosage volume and acted as a negative control. A third group of 5 animals were given 50 mg/kg bw of cyclophosphamide in bi-distilled water once at the same dosage volume and acted as a positive control.

Test substance:
LAS Batch Q-98.251.6 [purity 98.6%] was used as supplied.

Test substance formulation:
LAS was administered as a suspension in 0.5% carboxymethyl cellulose and 1% Tween 80 in bidistilled water.

Stability and formulation analysis:
The stability of the test substance, the test substance in the formulation and analysis of achieved concentration were not determined as part of this study.

Method preliminary study:
Two animals given LAS at the higher dose were sacrificed 48 hours post-administration. The bone marrow of both femurs was suspended in foetal calf serum and smears prepared. The slides were examined microscopically to assess their suitability. The test item, LAS (N^{α}-lauroyl-L-arginine), induced no toxicity at the high dose in males or females. In addition, 48 hours after treatment with LAS the analysis of the smears showed no toxic effects on the bone marrow. Therefore, the main study was carried out at a dose level of 2000 mg/kg using only male mice.

Method main study:
Bone marrow extraction: The mice were sacrificed 24 or 48 hours after administration. The bone marrow was removed from both femurs, suspended in foetal calf serum, centrifuged and the supernatant discarded. The pellet was resuspended in foetal calf serum and 3 slides per animal prepared.

Data collection: The slides prepared from each animal were analysed. Two thousand polychromatic erythrocytes (PCE) were counted for each animal and the number of micronucleated polychromatic erythrocytes (MNPCE) and the number of micronucleated normochromatic erythrocytes (NCE) were recorded. For each animal 1000 erythrocytes were counted and the ratio of PCE/(PCE+NCE) was determined in order to assess bone marrow toxicity. The micronuclei were identified according to the criteria established by Schmid[64,65].

Statistical analysis: Statistical analysis was performed on frequency of MNPCE/1000 PCE and the ratios PCE/(PCE+NCE) for each treatment group and sampling time. The results were compared with the negative control. ANOVA and Student's t test were used.

Systemic exposure study:
The systemic exposure to the test substance was determined on 2 occasions using 19 male mice of the same strain and body-weight range obtained from the same supplier as those used in the main study. Seven mice chosen from the animals received for the

[64] Krishna, G.; Hayashi, *M. Mutation Research*, 455, 155-166. 2000
[65] Schmid, W. *Mutation Research*, 31, 9-15. 1975

preliminary study and not treated previously were given the vehicle or the test item, LAS (N^{α}-lauroyl-L-arginine), at approximately 1700 mg/kg. The distribution of the animals into groups and the blood sampling times were as indicated below:

Treatment	Blood sampling time		
	0.5 hours	1 hour	3 hours
Negative Control	1	---	---
LAS (1700 mg/kg)	2	2	2

Subsequently 12 mice from the same supplier were given the vehicle or the test substance, LAS (N^{α}-lauroyl-L-arginine), at approximately 1700 mg/kg. The body-weight range of the animals on the day of administration was 25-35 g. The distribution into the different groups and the blood sampling times are indicated below:

Treatment	Blood sampling time		
	0.5 hours	1 hour	3 hours
Negative Control	2	2	2
LAS (1700 mg/kg)	2	2	2

Obtaining and storage of samples: Plasma samples were obtained from the treated animals at the designated sampling times on 2 occasions. The samples from each animal were centrifuged at 3000 rpm and the plasma collected. A pool of plasma from all the animals of each group was prepared at each sampling time, giving a total of 10 samples to be analysed. The samples were immediately frozen at –20 ± 5°C until their analysis. The samples were delivered to the Sponsor for analysis.

Determination of LAS levels in plasma: The determination of plasma levels of the product was carried out at Laboratorios Miret, S.A. (LAMIRSA) in November 2002 and was performed by High-Performance Liquid Chromatography (HPLC).

Results:

Preliminary study:

No mortality and no observable signs of toxicity were observed in the animals treated with 1300 or 1700 mg/kg bw of LAS. There were no differences in the toxic responses of males and females. Therefore, the main study was carried out in male mice at a dose level of 2000 mg/kg bw.

The bone marrow of the two animals that received the high dose level of LAS showed no toxic effects.

Main study:

No mortality or signs of toxicity were observed in the animals given LAS at a dose level of 2000 mg/kg bw. Similarly animals given the negative or the positive control substances showed no evidence of toxic effects.

There was no significant increase in the frequency of MNPCE/1000 PCE in the bone marrow of the LAS treated mice. Thus, LAS is not mutagenic under the experimental conditions of this assay. However, in animals given the positive control,

cyclophosphamide, at the dose of 50 mg/kg bw, there was a significant increase in the frequency of MNPCE/1000 PCE.

The toxic effects on the bone marrow of treated mice were assessed by comparing the values of the ratio of PCE/(PCE+NCE). No significant reduction in the ratio was observed when comparing the vehicle control and the groups given LAS at either sampling time. Comparing the negative control group with the positive control group, treated with 50 mg/kg bw of cyclophosphamide, no statistically significant reduction was observed although a slight decrease in the ratio was observed in the group given LAS and in the positive control group.

In the metabolism-toxicokinetic studies with lauric arginate (Appendix 17, 18), LAS was metabolised and transformed into arginine and lauric acid. It was expected that the oral administration of a single dose of LAS to mice would produce similar results with respect to the possible metabolism of the test substance. No LAS was detected in the plasma of the mice examined.

Conclusions:

When LAS was administered by oral gavage at a dose of 2000 mg/kg bw, there was no biologically significant increase in the frequency of MNPCE in the bone marrow of the treated mice at any sampling time. Therefore, LAS was considered to not be mutagenic.

Potential for intolerance and other adverse sensitive effects

The building blocks of lauric arginate are L-arginine HCl, lauric acid and ethyl alcohol. Once lauric arginate is ingested it is rapidly metabolised to arginine and endogenous products (Appendices 17-19, 21).

Human *in vitro* and *in vivo* studies have demonstrated that lauric arginate is immediately degraded to N^{α}-lauroyl-L-arginine (LAS) and thence to arginine (Appendices 19, 20, 22). There is no evidence in the literature that any of these substances are immunotoxic, allergenic nor are they suspected of causing food intolerance.

When lauric arginate was administered to rats continuously in the diet at levels of up to 50000 ppm (3714 and 3915 mg/kg bw/day for male and female rats respectively) in the subchronic (13-week) study (Appendix 10), and 18000 ppm (907 and 1128 mg/kg bw/day for male and female rats respectively) in the chronic (52-week) study (Appendix 11), or when Mirenat-N, a formulation of 20-25% lauric arginate, was administered to rats continuously in the diet at levels of up to 50000 ppm (3324 and 3927 mg/kg bw/day for male and female rats respectively) in the subchronic (13-week) study (Appendix 51), there was no increase in the incidence of infection and no evidence of tumour development in treated animals given lauric arginate or Mirenat-N. There were no effects of treatment on total white cell counts, or the differential cell counts, particularly lymphocytes, detected during routine investigations. There were no effects on the bone marrow, thymus, spleen, lymph nodes, Peyers patches and bronchus-associated lymphoid tissue in animals given either of the test substances.

Observed noisy and gasping respiration seen in the embryo-foetal toxicity studies in rats and rabbits were a consequence of oral gavage administration of a thick, irritant suspension of lauric arginate, which was accidentally aspirated by some treated animals (Appendices 12-15, 52-53).

After 13 and 52 weeks of continuous oral administration of lauric arginate at different dietary concentrations, an irritant effect on the stomach of a small number of rats was observed at the higher concentrations (Appendices 10, 11). These effects were slight and due to a local irritant reaction and not indicative of food intolerance. It should also be noted that when Mirenat-N, a formulation of 20-25% lauric arginate, was administered at dietary concentrations of up to 50000 ppm (3324 and 3927 mg/kg bw/day for male and female rats) for 13 weeks no irritant effects on the stomach were observed, confirming that this was a local reaction induced by very high dietary concentrations of lauric arginate (Appendix 51).

In the absence of effects at dietary levels of up to 50000 ppm (3714 and 3915 mg/kg bw/day for males and females respectively) specific testing for immunotoxicity, allergenicity and potential food intolerance was considered unnecessary.

Nutritional considerations

The following table reports the nutritional considerations of a sample of formulated lauric arginate, Mirenat-N, a formulated product used as the test substance applied in food products. Test substance: Mirenat-N Export (Batch Number 14730, 10% of purity) (Appendix 57).

Table 18: *Nutritional parameters of lauric arginate*

Determination	Result	Procedure
Fatty Acids	0.75 %	PNT 2/07/002
Total Carbohydrates	n.d.[66]	PNT 2/08/006
Sodium	970.8 ppm	PNT 4/19/003/B
Dietary fibre	n.d.	PNT 4/06/004
Sugars	n.d.	PNT 2/01/010
Sugar alcohol	n.d.	HPLC
Proteins	8.85 %	PNT 2/16/006/G
Chlorides (as NaCl)	1.86 %	PNT 2/03/011
Humidity	87.63 %	PNT 2/08/003/G
Ashes	2.28 %	PNT 2/03/008
Sulphites	Negative	PNT 2/19/011/B
Chromatography of fatty acids:		
C12 Lauric acid	99.04 %	PNT 3/01/011
C13 Tridecanoic acid	0.30 %	PNT 3/01/011
C18:1 Oleic acid	0.17 %	PNT 3/01/011
C18:2 Linoleic acid	0.50 %	PNT 3/01/011
Saturated fatty acids	99.34 %	PNT 3/01/011
Mono-unsaturated fatty acids	0.17 %	PNT 3/01/011
Poli-unsaturated fatty acids	0.50 %	PNT 3/01/011
Energetic value	42.15 Kcal/100 g	PNT 3/01/011
Energetic value	178.20 Kj/100 g	PNT 3/01/011

[66] n.d.: non detected

Human Data

Human *in vivo* studies investigated the metabolism of the active ingredient of lauric arginate (ethyl-N^{α}-lauroyl-L-arginate HCl) in humans (Appendix 20).

Adult male volunteers received a single oral dose of 1.5 or 2.5 mg/kg bw ^{13}C-ethyl-N^{α}-lauroyl-L-arginate HCl as a 25% solution in propylene glycol. These doses were calculated to be in the range of the expected human exposure of 3 mg/kg bw/day (high level intake) or 1 mg/kg bw/day (mean average intake).

In a series of blood samples taken after administration, the active ingredient of lauric arginate was not detected due to its hydrolysis to its metabolites (LAS, arginine and lauric acid). The concentration of the metabolites declined to non-detectable levels within 12 hours demonstrating that ethyl-N^{α}-lauroyl-L-arginate HCl is hydrolysed into endogenous compounds. In addition, there were no significant abnormalities in any of the laboratory or clinical data for either of the two oral doses.

Safety Reports prepared by International Agencies

No safety reports issued by regulatory authorities in other countries or by any international agencies are currently available.

Dietary Exposure to the Food Additive

Food Groups to Contain Food Additive

Table 19*: Maximum Proposed Level/Concentration of Food Additive in Foods*

Item	***Food types***	***Lauric arginate (ppm; maximum)***
0.1	Preparations of food additives	225
1.6	Cheese - soft/cream/processed and mozzarella	450
1.6	Cheese – Hard/Semi-hard	1 mg/cm^2 of surface area of cheese.
1.4.2	Cream products (flavoured, whipped, thickened, etc)	225
2.2.2	Oil emulsions (<80% oil)	450
3	Ice confection sold in liquid form excluding milk based ice confections	225
4.1.3	Peeled and/or cut fruits and vegetables	225
4.3.1	Dehydrated legumes	225
4.3.4	Low joule jams	225
5	Confectionery products (e.g. chewing gums) but excluding chocolate and cocoa products	5625
6.4	Flour products (including noodles and pasta)	225
7	Breads and bakery products (surface treatment only)	450
9.3	Fish products	450

Item	***Food types***	***Lauric arginate (ppm; maximum)***
10.2	Liquid egg products (yolk preparations only)	450
11.4.1	Tabletop sweeteners – liquid preparation	115
12.6	Vegetable protein products	225
13.1	Infant formula products (in powder form)	2250
13.4	Formulated supplementary sports drinks	55
14.1.2	Fruit and vegetable juices and fruit and vegetable juice products (NOT apple juice)	55
14.3	Alcoholic beverages not included in item 14.2	55
14.1.3	Water based flavoured drinks and high energy drinks and soft drinks	55
14.1.5	Tea, herbal infusions and similar products (ready-to-drink only)	55
8.2, 8.3	Meat and meat products, including poultry	450
20.1	Beverages	55
20.2	Savoury toppings or fillings - essentially sauces such as tomato paste used in ready to eat pizzas, etc.	225 (vegetables) 450 (cheese)
20.2	Dairy and fat based desserts, dips and snacks Soup bases (made up as directed)	450

Percentage of Food Group in which Food Additive is likely to be used

All commodities listed could have lauric arginate added with the following exceptions:

1. Fruit juices and drinks: Lauric arginate will not be used in pasteurised drinks, drinks that claim "no additives" and organically certified drinks. It is estimated that less than 50% of fruit juices will be likely to incorporate lauric arginate and up to 75% of fruit juice drinks.
2. Surface treatment of hard and semi-hard cheese: Lauric arginate will not be incorporated within these types of cheese. It will be placed on the outside of the cheese. Studies have shown that that lauric arginate does not penetrate more than 3mm below the surface of solid cheeses.

Use of the Food Additive in Other Countries

Authorizations in USA

In December 2004, the Food Drug Administration (FDA) approved lauric arginate as the common and usual name for ingredient labeling purposes.

On 1st March 2005, Grupo LAMIRSA submitted a GRAS (Generally Recognised As Safe) Notice for lauric arginate to the FDA who designated this document as GRN number 000164 (Appendix 58). A complementary document was also submitted to United States Department of Agriculture (USDA) in order to demonstrate the efficacy of lauric arginate for meat and poultry products.

The following table lists the intended food uses of lauric arginate submitted as the basis of the GRAS Notice, classified in terms of the food categories listed for the purpose in the US Code of Federal Regulations [21 CFR 170.3 (n)]:

Intended food uses of lauric arginate according to 21 CFR 170.3(n)

Up to 200 ppm ethyl-N$^{\alpha}$-lauroyl-L-arginate hydrochloride[67] in the following food categories
(3) Beverages and beverage bases, not including dairy products, soft drinks, or alcoholic beverages.
(3) Carbonated beverages[68]
(5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses.
(7) Tea, including regular, decaffeinated, and instant types.
(8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs.
(11) Egg dishes including egg roll, egg foo young, egg salad, and frozen multicourse egg meals, but not fresh eggs.
(12) Margarine and margarine-like table spreads, mayonnaise, and spoonable and pourable dressings for salads.
(13) Fish products, including all prepared main dishes, salads, appetizers, frozen multicourse meals, and spreads containing fish, shellfish, and other aquatic animals, but not fresh fish.
(15) Fresh fish, including only fresh and frozen fish, shellfish, and other aquatic animals.
(17) Fresh meats, including only fresh or home-frozen beef or veal, pork, lamb or mutton and home-prepared fresh meat-containing dishes, salads, appetizers, or sandwich spreads made therefrom.
(18) Fresh poultry, including only fresh or home-frozen poultry and game birds and home-prepared fresh poultry-containing dishes, salads, appetizers, or sandwich spreads made therefrom.
(22) Pie fillings.
(24) Gravies and sauces, including all meat sauces and gravies, and tomato, milk, buttery, and specialty sauces.
(29) Meat products, including all meats and meat-containing dishes, salads, appetizers, frozen multicourse meat meals, and sandwich ingredients prepared by commercial processing or using commercially processed meats with home preparation.
(34) Poultry products, including all poultry and poultry-containing dishes, salads, appetizers, frozen multicourse poultry meals, and sandwich ingredients prepared by commercial processing or using commercially processed poultry with home preparation.
(35) Processed fruits and fruit juices excluding apple juice, including all commercially processed juices and juice punches, concentrates, dilutions, ades, and drink substitutes made therefrom, and dried fruits; also including strained fruits and fruit juices excluding apple juice as baby or toddler foods.
(36) Processed vegetables and vegetable juices, including potato salads, raw vegetables, vegetable juices and blends, and tomato sauces
(40) Soups and soup mixes, including commercially prepared meat, fish, poultry, vegetable, and combination soups and soup mixes.

[67] Equivalent to 225 ppm as lauric arginate.
[68] The maximum concentration of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl is 100 ppm

On 1st September 2005, the FDA issued a Letter of No Objection regarding the status of lauric arginate as GRAS for use as an antimicrobial agent in the above food categories at levels up to 225 ppm of lauric arginate (Appendix 58).

In June 2008, an Expert Panel agreed that adding lauric arginate to chewing gum at a level of 0.5% of the active ingredient is GRAS.

On 21st September and 13th October 2005, USDA issued two letters that determined the efficacy and suitability of lauric arginate for use in meat and poultry products (Appendix 59, 60). These letters are summarised in the FSIS Directive 7120.1 along with recent approvals that concern the use of lauric arginate with other solvents to treat comminuted meat products and fresh cuts of meat and poultry (Appendix 61). This document can be found in the following website:
http://www.fsis.usda.gov/regulations_&_policies/ingredients_guidance/index.asp

Authorization in Mexico

On 17th July 2006, the Heath Secretary of Mexico (Secretaria de Salud) published in its Official Journal ("Acuerdo por el que se determinan las sustancias permitidas como aditivos y coadyuvantes en alimentos, bebidas y suplementos alimenticios") (Appendix 62) that lauric arginate is an allowed substance to be used as a food additive for human consumption.

Authorizations in Europe

Food Authorisation

On 13th March 2006, the Company requested European Union authorization of lauric arginate as a new additive for use in food for human consumption. The European Commission has requested to European Food Safety Authority to provide a scientific opinion on the safety in use of lauric arginate as a food preservative. EFSA issued in April 2007 the opinion of the Scientific Committee on lauric arginate as a new food additive with the reference number EFSA-Q-2006-35 (Appendix 63)

Assessment Procedure

The applicant proposes that this application for approval of a food additive that is not currently permitted be considered using the following procedure:

- General procedure Level 2.

Exclusive Capturable Commercial Benefit (ECCB)

Laboratorios Miret, S.A., the applicant for this application acknowledges they will derive exclusive capturable commercial benefit as a result of the application being approved and the Standard being modified as a result. A statutory declaration to this effect is enclosed (Appendix 64).

International and Other National Standards

International Standards

Authorization in JECFA

On 24th-28th April 2006, during the *38th session of the Codex Committee on Food Additives and Contaminants*, LAMIRSA, proposed adding adding lauric arginate in the Priority List of Food Additives, Contaminants and Naturally Occurring Toxicants proposed for evaluation by JECFA (Appendix 55). JECFA reviewed the proposal in June 2008 during the 69th Session of the Joint FAO/WHO Expert Committee on Food Additives and allocated and ADI for lauric arginate of 4 mg/kg bw/day.

European List of Notified Chemical Substances (ELINCS): According to European Directive 67/548/EEC, Grupo LAMIRSA notified lauric arginate as a new substance in 2000. The registration number of the dossier was 00-11-0173 and L.A.E. was designated as the trade name of lauric arginate (Appendix 66).

Lauric arginate was notified according to Annex VIIA under the use category 005 personal/domestic and under the following desired effects: 015 cosmetic; 007 anti-static agents and 050 surface active agents.

The EC number assigned to lauric arginate is 434-630-6 included in the European List of Notified Chemical Substances (ELINCS).

Statutory Declaration

A statutory declaration has been enclosed with the application (Appendix 69).

Checklist

A Checklist is enclosed with the application (Appendix 70).