

CALCIUM L-THREONATE: A BRIEF REVIEW

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ABSTRACT

Introduction: Osteoporosis is a common metabolic bone disease. About 90% of osteoporosis is primary even though it may be caused by a number of other diseases. With the age increasing or menopause, the mineral substances and matrix of bone disease which result in a change of the microstructure of bone tissue and thus deviates the normal loading functions of bones and markedly increases the risk of fracture and also brings about systematic bone pain and changes of body attitude. Attempts have been done to treat osteoporosis with a variety of pharmacological agents, such as estrogens. Owing to the complexity of the causes of osteoporosis, there is evidence to show therapies (such as administration of calcium carbonate) are not effective for preventing osteoporosis. **Methods:** Calcium L-Threonate as a more efficient source of calcium will be researched and reviewed using the quantitative technique of meta-analysis. A total of 38 eligible papers, were identified in the literature. **Discussion:** Calcium L-Threonate is the calcium salt of L-threonic acid, one of the major metabolites of vitamin C produced during the metabolic process in vivo. Pre-clinical studies show that calcium L-Threonate improves the activity and functioning of osteoblasts and osteocytes while increasing intestinal absorption of calcium and enhances calcium utilization in bones to ensure optimized restoration and rebuild of bones. In animal studies, the bioavailability of calcium from this source was higher than from other sources of calcium. Calcium L-Threonate has low oral acute toxicity, with no adverse effects observed. In sub-chronic studies with Calcium L-Threonate, effects on blood coagulation time and accretion of the thyroid gland were identified. The effects on blood coagulation time and the thyroid gland were reversible and that a mild accretion in the thyroid gland in

rats was limited to males only. Reproductive and developmental toxicity studies in mice indicated that calcium L-Threonate in doses up to 6 g/kg bw/day has no adverse effect on the fertility and the developing foetus, and did not cause maternal toxicity. **Conclusion:** Consistency of findings suggests that calcium L-Threonate is more bioavailable, better absorbed and efficient calcium source, without the need of vitamin D presence.

INTRODUCTION

Research has shown that if animals are deprived of calcium that osteoporosis can occur, additionally, in humans it has been demonstrated that an increase in calcium intake can lead to a decrease in hip fracture rates [1]. Bones are of course made up of calcium and other minerals, so it stands to reason that an increase in dietary calcium will lead to bone strengthening. According to recent figures from the National Osteoporosis Society (NOS), in the UK, one in two women and one in five men over the age of 50 will break a bone mainly because of poor bone health. Osteoporosis costs the NHS and government £2.3 billion a year – that's £6 million a day. There are about 230,000 osteoporotic fractures every year and 1,150 people are dying every month in the UK as a result of hip fractures [2]. This paper applies meta-analytic techniques to a review of the literature on a new approved source of calcium, Calcium L-Threonate. The aim was not only to produce a single summary measure of the effect of Calcium L-Threonate in the treatment of Osteoporosis but also to make a direct comparison between the use of well known calcium supplements and this new approved calcium source. As Calcium L-Threonate is a new source of calcium, this work provide a brief familiarisation with the chemical compound. Proceeded by a discussion on how Calcium L-Threonate provides considerable advantages over traditional calcium supplements at biological, toxicological and pharmacological level. This report concludes with a summary of the implications that these results have for future research and current public health action.

DISCUSSION

Vitamin C is known for its biological activity and complicated mechanism of action. Studies show that vitamin C metabolites are likely to have physiological actions or, otherwise, have an impact on the physiological actions of vitamin C, which indicates the need for further research of these metabolites. Being a principal substance produced during the metabolism process of vitamin C, L-threonic acid (2R,3S-2,3,4-trihydroxy-butyric acid) can be found in the urine of human being as well as in most of higher animals and plants. Findings of these studies demonstrate active physiological actions of L-threonic acid, such as being the substrate of biosynthesized branched-chain amino acid like L-threonine. Calcium L-Threonate, the calcium salt of L-threonic acid, was reported to be synthesized in laboratory in 1979 as an oxidation product of vitamin C with hydrogen peroxide. In 1989, Dr. Verlangieri in his study of the pharmacological action of Calcium L-Ascorbate discovered calcium L-Threonate, produced during the preparation process of L-ascorbic acid and present in the product with a content of generally 3% because of oxidation of vitamin C in the air, enhanced vitamin C absorption and prolong its pharmacological action in the metabolic process. Further experimental research confirmed the above-mentioned function of calcium L-Threonate. Dr. Verlangieri thus called calcium L-Threonate metaphorically as the "door opener" to facilitate absorption of vitamin C. The follow-up studies revealed that calcium L-Threonate had a better effect in increasing the vitamin C level in fibroblasts than calcium chloride in the control group. However, there was no report of L-threonic acid or its derivatives being used as medicaments at that time. By virtue of chiral identification present in creatures, chiral medicaments received extensive concerns. L-threonic acid has a simple optical structure of L shape as vitamin C, yet manifests an active biological activity, thanks to its chiral structure. Verlangieri's study revealed the important effect of L-threonic acid on the physiological function of calcium L-Threonate. Generally, calcium supplements in both domestic and international markets are mainly: (1) calcium salts of mineral acid, such as calcium carbonate, calcium oxide (active calcium), calcium chloride and calcium phosphate etc.; (2) calcium salts of organic acid, such as calcium Gluconate, calcium citrate, calcium malate, calcium lactate and calcium aspartic acid; and (3) multi-calcium tablets combining several kinds of calcium salts. The key point in calcium supplement research is to achieve better intestinal absorption of calcium, and consequently, enhance efficiency and quality of calcium utilization in bones. The purpose is to realize efficient and high-quality calcium utilization. Under general conditions, calcium

doesn't exist but in the form of calcium salts. Numerous experiments indicate different physico-chemical properties of calcium salts with different acid radicals. These calcium salts are distinct from each other in terms of absorption and utilization. Therefore, the selection of acid radical is essential for improving calcium absorption and utilization. Taking into consideration the research of concerning vitamin C, L-threonic acid and calcium L-Threonate and the findings thereof, a comprehensive research was launched into calcium L-Threonate as a new calcium supplement.

BIOLOGICAL & TOXICOLOGICAL DATA

Absorption

Studies have shown that to be nutritionally effective the calcium in the calcium L-Threonate must be absorbed through the intestine into the bloodstream. The different sources of calcium exhibit different dissolution characteristics and these affect both the biochemical and physiological aspects giving differences in the methods and rates of absorption of the element from its source. The absorption of calcium from the intestine involves both active and passive processes. The former requires energy (as ATPase) and is of limited duration, whilst the latter is based on diffusion, is continuous and does not require energy.

Study on the Absorption of Calcium L-Threonate

In order to assess the relative contributions of the active and passive processes in the absorption of calcium from the Threonate a study was carried out on rats. This study was designed to compare three organic calcium salts: calcium Gluconate, calcium acetate and calcium L-Threonate. For each calcium source there were two groups, each consisting of 6 male Wistar rats. One group was administered an ouabain-Tyrode solution in addition to the test substance. The ouabain was used to block the ATPase and thus the active absorption stage. The other group was able to exhibit both active and passive absorption. The relative passive absorption ratio of the calcium was calculated from the quotient of the absorption value of the two groups. The serum calcium concentration in the blood was determined for each group at intervals of 15, 30, 60 and 90 minutes after administration. The results were evaluated on the basis that the ouabain group had the active absorption inhibited permitting only passive absorption whereas both active and passive absorption took place in the other groups. The study showed that at the same calcium dose at any given time point, the value for the serum calcium from the calcium L-Threonate treated animals was higher than that for either the calcium Gluconate or calcium acetate groups under the same conditions. It was also found that the total absorption of the

calcium from the Threonate was greater but also the proportion of its passive absorption. (Table 2)

Table 2: Concentration increase of blood calcium after intestinal administration *in vivo* (µg/ml) n=6

Group	15min	30min	60min	90min	
Calcium acetate		25.97	48.18	32.43	
	Buffering solution	± 22.13	± 14.66	± 11.76	17.70 ± 9.69
	Ouabain	± 10.44	± 15.89	± 13.02	8.87 ± 3.64
	Passive absorption (%)	40%	33%	40%	50%
Calcium Gluconate		13.73	43.76	24.88	10.67
	Buffering solution	± 2.06	± 14.95	± 3.23	± 7.92
	Ouabain	9.7 ± 4.2	25.50 ± 6.91	12.63 ± 4.35	8.43 ± 4.8
	Passive absorption (%)	70.6%	58%	51%	50.9%
Calcium L-Threonate (high dose)		31.65	86.38	58.81	23.6 ± 17.63
	Buffering solution	± 15.24	± 14.14	± 29.08	
	Ouabain	29.32 ± 8.43	69.72 ± 14.03	32.76 ± 18.34	12.18 ± 6.32
	Passive absorption (%)	93%	78%	56%	52%
Calcium L-Threonate (middle dose)		24.91	42.05	19.01	14.80
	Buffering solution	± 8.91	± 6.85	± 8.55	± 8.80
	Ouabain	20.51 ± 3.58	30.96 ± 9.33	10.74 ± 5.25	5.26 ± 4.13
	Passive absorption (%)	82%	74%	56%	36

The higher rate of passive absorption of calcium L-Threonate is significant. With regard to the total absorption of the calcium L-Threonate it was shown that passive absorption was the main route of transfer; not only as the passive diffusion of the calcium ion but also as a passive diffusion process for the calcium L-Threonate molecule.

BIOAVAILABILITY

Absorption of Calcium L-Threonate (Rodent Study)

The absorption rate, absorption capacity and availability of the calcium L-Threonate were studied in rats and compared to calcium carbonate. Thirty SD rats (half female, half male) were randomly divided into three groups of 10 with 5 females and 5 males in each group. The groups were assigned as:

1. Control
2. Calcium L-Threonate administration
3. Calcium carbonate administration

The rats were kept individually in metabolic cages and given standard rat feed for the first three days and then converted to a calcium depleted feed. On the seventh day the rats had doses of the calcium sources adjusted to deliver 40mg / kg / day of calcium injected into the stomach. The control group was injected with an amylum solution. Animals of each group were allowed to drink deionised water *ad lib*. Urine and faeces were collected 24 hours after the calcium administration. An EDTA titration was used to evaluate the calcium content of the urine and faeces. The results of the calcium absorption are given in Table3. The rates were calculated from the data as:

a)
$$\text{Percentage Absorption rate} = \frac{\text{ingested calcium} - \text{calcium in faeces}}{\text{ingested calcium}} \times 100$$

b)
$$\text{Percentage availability} = \frac{\text{ingested calcium} - \text{calcium in faeces} - \text{calcium in urine}}{\text{ingested calcium} - \text{calcium in faeces}} \times 100\%$$

c)
$$\text{Net availability} = \text{absorption rate} \times \text{availability}$$

d)
$$\text{Absorption capacity} = \text{net availability} \times \text{ingested calcium}$$

Table 3: Absorption and utilization of calcium L-Threonate in rats

It was concluded that the absorption rate, availability and absorption capacity of the calcium L-Threonate was significantly higher than the equivalent values obtained for calcium carbonate.

Absorption and Utilisation of Calcium L-Threonate in Rats

The study was carried out with the objective of investigating the rates of calcium absorption and utilisation in rats. 30 Male SD rats were randomly divided into three groups of 10 after 3 days observation. Each animal was kept in a single cage. The substance administrations to the group were:

1. Control group:- 3% amyllum suspension
2. Test group 1 (high dose):- Threonate-amyllum suspension containing 80mg calcium.
3. Test group 2 (low dose):- Threonate-amyllum suspension containing 40mg calcium.

Calcium deficiency models were induced by subjecting the rats to a low level of calcium intake following three days of feeding with a calcium containing feed. From the fourth day, in addition to the low calcium feed the rats were administered the dosages relevant to their group. During the observation period the animals were given distilled water and all urine and faeces were collected. Environmental conditions were maintained at a temperature of 20°C ± 1°C, 55% humidity and 12 hrs / day illumination. The calcium concentration of blood, urine, faeces and feed was recorded for each animal before and after the investigation. During the study the animals exhibited normal behaviour, there was no change in appetite and they showed normal weight gain. No deaths occurred. The results were calculated from the data as:

- a)
$$\text{Percentage Absorption rate} = \frac{\text{ingested calcium} - \text{calcium in faeces}}{\text{ingested calcium}} \times 100$$
- b)
$$\text{Percentage availability} = \frac{\text{ingested calcium} - \text{calcium in faeces} - \text{calcium in urine}}{\text{ingested calcium} - \text{calcium in faeces}} \times 100\%$$
- c)
$$\text{Net availability} = \text{absorption rate} \times \text{availability}$$

Table 4: Absorption and utilization of calcium L-Threonate in rats

Absorption rate (%)	Availability (%)	Net availability (%)
(a)	(b)	

Group	Rats	Absorption rate (%) (a)	Availability (%) (b)	Net availability (%) (c)	Absorption capacity / day in mg (d)
Calcium L-Threonate	10	94.07 ± 2.24	93.5 ± 7.3	88.6 ± 6.1	33.4 ± 2.5
Calcium carbonate	10	44.49 ± 4.49	90.3 ± 9.7	33.7 ± 5.7	13.5 ± 2.9
(c)					
High dose (n=10)		95.30 ± 3.47	76.83 ± 7.58	73.06 ± 6.16	
Low dose (n=10)		94.33 ± 6.12	89.13 ± 3.98	84.11 ± 7.28	
Mean value		94.82	82.98	78.58	

Table 5: Absorption of calcium in rats from Calcium L-Threonate

	Calculated net absorption of calcium (mg)	Absorption in mg / kg body weight
High dose (n = 10)	58.60 ± 4.93	293
Low dose (n = 10)	33.64 ± 2.91	168
Mean value	46.12	230

In terms of the plasma calcium concentration, that of both the administration groups was found to be higher than in the control group. (Table 6).

Table 6: Plasma calcium concentration (mg/dl)

	Before administration	After administration
High dose (n = 10)	13.98 ± 3.72	16.125 ± 1.70
Low dose (n = 10)	14.51 ± 4.06	14.92 ± 1.11
Control group (n = 10)	13.98 ± 3.56	13.44 ± 0.76

Whilst the levels of calcium determined from the faeces was very similar for both groups, the calcium in the urine was very much higher in the high dose rats. (Table 7)

Table 7: Excretion of calcium in urine and faeces after dosing

	Total Ca in urine/day (mg)	Total Ca in faeces/day (mg)
High dose (n = 10)	17.79 ± 3.30	3.76 ± 2.98
Low dose (n = 10)	3.86 ± 1.63	4.27 ± 2.43

The results of the study indicate that there were no significant differences between the absorption rates at the two dose levels. However, the rate of utilisation was higher for the low dose group than the higher dose group. The plasma calcium concentrations of both dosage groups were higher than the control with the high dosage group having the higher concentration. The net utilisation of the calcium was over 70% in both groups and the results indicate that the low dose group had the higher net rate. During the study no adverse effects were observed in any of the rats in both dosage groups.

Study using ⁴⁵Ca – Labelled Tracer

A study of the pharmacokinetics of calcium L-Threonate was carried out using a ⁴⁵calcium tracing technique on rats. The ⁴⁵calcium L-Threonate was prepared from ⁴⁵calcium chloride. 18 rats (9 male and 9 female) were randomly divided into three

groups. Each group was allocated a dosage corresponding to either 100mg Ca / kg body weight, 200mg Ca / kg body weight or 300mg Ca / kg body weight. The doses were administered orally after the rats had fasted for 16 hours. Blood was taken from the ophthalmic artery of the rats at 0.25, 0.5, 1.0 and 1.5 hours after administration and then at 2.0, 3.0, 4.0, 6.0, 8.0, 12.0 and 24 hours. Tissue was removed from muscle, femur, brain, lungs, heart, liver, spleen, kidney, stomach and intestines at 0.5, 1.0, 4.0 and 8.0 hours after dosing. Urine and faeces were obtained at 0-2, 2-4, 4-6, 6-8, 8-12 and 12-24 hours. The calcium content of the samples (as µg Ca) was evaluated by the radioactive count rate (min⁻¹) [activity / ml], dilution rate and specific activity of the ⁴⁵calcium L-Threonate, using a liquid scintillometer. The results showed a positive correlation between the increase in plasma calcium and the dosage of calcium L-Threonate. The pharmacokinetic parameters studied (A, Ke, Ka, T₁₂ (KE), T₁₂ (Ka), T max, C max, AVC, CL/F(S) and V/F(C)), also showed positive correlations with dosage at all doses. The concentration-time curve at all doses had a similar trend. The calcium levels in the heart, liver, spleen, kidney and brain tissues were not increased significantly. However, a significant increase in calcium was found in the sternum and femur (Table 8).

Table 8: Incremental increase of calcium in tissue from oral administration(x ± s, n=6)

Tissue	Increment of calcium in tissue /µg·g ⁻¹			
	0.5h	1.0h	4h	8h
Heart	3.64 ± 0.94	3.74 ± 0.73	2.82 ± 0.31	4.25 ± 0.31
	4.27 ± 0.45	4.64 ± 0.27	3.55 ± 0.44	2.97 ± 0.49
Liver	2.89 ± 0.19	5.14 ± 0.79	2.72 ± 0.26	3.20 ± 0.16
	28.44 ± 6.86	33.45 ± 9.03	10.65 ± 2.36	15.39 ± 6.12
Spleen	24.95 ± 4.22	37.62 ± 8.76	47.41 ± 12.67	29.30 ± 2.42
	4.36 ± 0.65	8.75 ± 1.10	4.90 ± 0.64	3.86 ± 0.79
Stomach	0.68 ± 0.12	1.54 ± 0.41	1.52 ± 0.24	2.62 ± 0.29
Intestines				
Kidney				
Brain				

Sternum	10.41 ±	16.52 ±	36.56 ±	42.77 ±
	1.27	3.09	9.31	5.17
Femur	21.08 ±	27.44 ±	29.94 ±	37.53 ±
	4.84	2.44	7.84	3.60

An increased concentration of calcium was also found in the jaw, incisor and molar teeth of mature rats which indicate that calcium metabolism also took place in mature tissue. (Table 8).

Table 9: Content of calcium deposition in rats bones(x ± s, n=6)

Dose/mg(Ca)·kg ⁻¹	Bone	Content of calcium deposition /μg·kg ⁻¹ (Bone)			
		1.0h	4.0h	24.0h	
100	Sternum	2.11 ±	3.59 ±	30.02 ±	
		0.34	0.45	2.61	
	Femur	8.18 ±	9.41 ±	36.27 ±	
		1.75	0.72	4.86	
	Teeth	1.68 ±	2.96 ±	26.38 ±	
		0.41	0.56	4.12	
	Jaw	2.44 ±	4.37 ±	23.48 ±	
		0.52	1.24	5.12	
	200	Sternum	16.52 ±	36.56 ±	39.09 ±
			3.09	9.31	3.67
		Femur	27.44 ±	29.94 ±	48.67 ±
			2.44	7.84	8.32
Teeth		4.99 ±	14.41 ±	38.18 ±	
		1.82	1.52	7.63	
Jaw		5.12 ±	17.19 ±	47.23 ±	
		1.07	4.76	10.96	
300		Sternum	18.21 ±	28.64 ±	40.48 ±
			4.89	2.24	4.69
		Femur	9.38 ±	33.02 ±	49.62 ±
			2.94	4.65	4.66
	Teeth	6.84 ±	20.33 ±	33.46 ±	
		1.28	4.64	6.24	
	Jaw	12.67 ±	24.56 ±	38.80 ±	
		3.85	5.32	5.76	

The observation that significant quantities of calcium were deposited in the bones and teeth 24 hours after administration supports the supposition that calcium L-Threonate can be beneficial as a calcium supplement.

Further Bioavailability Studies

Studies on mice and rats have indicated that calcium L-Threonate can enhance the levels of blood calcium, bone density and bone calcium content, and decrease serum alkaline phosphatase and bone reabsorption. When compared to calcium Gluconate or Caltrate Plus D the positive effects, and hence the bioavailability, of calcium L-Threonate was found to be greater than that of either of the other two sources of calcium. A further study on bone sedimentation in mice using radioactive calcium indicated that the calcium bioavailability of calcium L-Threonate was higher than that for Caltrate Plus D. Studies investigating the total human urinary content of calcium and creatine following administration of calcium L-Threonate have indicated that calcium L-Threonate appears to rapidly increase calcium reserves in the human body. The effects of calcium L-Threonate were greater than for calcium citrate, calcium Gluconate and calcium carbonate, suggesting that calcium L-Threonate is more readily absorbed by the human body.

KNOWN INTERACTIONS OF CALCIUM L-THREONATE WITH OTHER COMPONENTS IN THE DIET

Studies undertaken on calcium L-Threonate have not revealed any adverse interactions of calcium L-Threonate with other components in the diet.

IMPACT OF CALCIUM L-THREONATE ON THE INTESTINAL MILIEU AND ON THE ABSORPTION OF OTHER NUTRIENTS.

Numerous toxicological, pharmacological and pharmacokinetic studies have not revealed any adverse effect of calcium L-Threonate on the intestinal milieu, nor does there appear to be any competitive absorption with, or other affect on, other nutrients.

Test for Induction of Chromosomal Aberrations

A chromosomal aberration test was carried out on calcium L-Threonate using cells harvested from Chinese hamsters. Calcium Threonate dosage was selected from a pre-trial study in which a dose of 2.50mg / ml resulted in 50% of the cells manifesting growth inhibition. The test doses derived from this study and used for the test were 2.5mg / ml, 1.25mg / ml and 0.62mg / ml. Cells were harvested at 24 and 48 hours. The results of the test were that the cells cultured with calcium L-

Threonate showed no significant difference between the dosage and control group with respect to the rate of chromosomal aberration whereas a significant difference was observed between the positive group and the control group. It was concluded that the calcium L-Threonate had no teratogenic action on the chromosomes of Chinese hamster cells.

Reproductive Toxicity

A study was carried out to observe whether embryotoxicity or teratogenesis occurs in mice administered with the calcium L-Threonate for a continuous period of 10 days during the early stages of pregnancy of the mice. Details and results of the study are given in Annex. The test substance was administered in four dose groups, 6g / kg / day, 4g / kg / day and 2g / kg / day and a control of 20ml / kg / day of sodium carboxymethyl cellulose suspension. Administration took place from the 6th to the 15th day of pregnancy for 10 days. The mice body weights were checked at 0, 3, 7, 10, 13, 16 and 20 days. The results showed no effect of the calcium L-Threonate on the body weights of the mice during pregnancy. There was no significant difference ($P > 0.05$) between the body weight increases of the three dose groups and the control group. Normal bodily functions and behaviour were observed in all the mice. In terms of foetal growth and development there was no significant difference ($P > 0.05$) in the dead foetus rate between the test and control groups. No malformation was observed in the living foetuses (head, face, limbs, tail and trunk). Post-mortem tests on bone specimens showed no malformation of the bones in any group and no aberrations / malformations were observed in the foetal internal organs. It was concluded that, under the conditions of the test, calcium L-Threonate did not induce teratogenesis in pregnant mice.

Perinatal / Postnatal Development Study

Calcium L-Threonate was administered by gavage at dose levels of 6g / kg / day, 4g / kg / day and 2g / kg / day to pregnant Kunming mice from the 15th day of pregnancy to weaning. The results indicated that there was no significant difference ($P > 0.05$) in the body weight increases between each administration group and the control group. There was no toxicity observed in the parent mice. The mice in all the groups gave birth after 20 – 21 days pregnancy with litter sizes ranging from 6 – 12 and no significant difference between the control and administration groups. The gavage administration of the same dosage levels were continued in the parent mice during lactation. The survival rates for every group was 100%. After the weaning the growth and development of the newborn mice were rated as normal indicating that the administered substance had no toxic effect on the growth and

development of the newborn mice. For the F1 mice in all groups the physiological development maturing indices showed no abnormalities and the neurological development indices indicated that the calcium L-Threonate had no marked toxicity on the nervous systems of the F1 mice. Behavioural studies in the form of a net-climbing test showed no significant difference between each of the administration groups and the control groups. A coordination test using the rotating rod method also indicated that there was no significant difference between the groups. In addition, no significant difference ($P > 0.05$) was observed between the test and control groups on the effect of the hypnosis dose under mebumal sodium threshold; swimming breakdown duration terms and on learning and memory capability in the F1 mice. A sample of the ten 60 day old F1 mice from each group was mated in the male : female proportion of 1 : 1. No significant difference in the mating behaviour of the mice in each group was observed and there was no significant difference in the pregnancy rates and the newborn mice body weights. Surface checks on the F2 foetuses showed no abnormalities. From the study it was concluded that, under the test conditions, calcium L-Threonate had no reproductive toxicity in any administration group. No surface malformations were observed on the F1 mice and there was no significant effect of the administration on the growth and development, neurophysiological development, neural behaviour and reproductive function of the mice.

Reproductive Toxicity Study on Mice

A reproductive toxicity study was carried out using Calcium L-Threonate as the test substance on Kunming species mice. The details of the study are given in Annex. A total of 80 virgin female mice weighing 25 – 27g and 80 male mice weighing between 18 and 20g were divided randomly into four groups on the basis of their body weights. The four groups were assigned three dosage levels of calcium L-Threonate and a control group received a sodium carboxymethylcellulose suspension. The high dose group received 6g / kg / day calcium L-Threonate, the middle group 4g / kg / day and the low dose group 2g / kg / day. Administration was by gavage, with the males receiving the dose for 60 continuous days and the virgin females for 14 continuous days. The mice were then mated in the same cage in the proportion of male to female of 1 : 1, 24 hours after the last administration to each sex. Administration by gavage was then continued with the female mice at the previous dosage levels. The mice were weighed every week during the administration period and dosage adjusted to body weight. The pregnant mice were killed on the 21st day of their pregnancy and subjected to an anatomical investigation. This included pregnancy rate, number of living foetuses, number of dead

foetuses, weights of the living foetuses, egg implantation numbers and the number of resorptive foetuses. The results showed that the body weights increased every week during the administration period and that good growth and development was observed. There were no visible signs of toxicity. Post mortem anatomical inspection of the pregnant mice showed there were no malformations to the appearance of the mice or foetuses and none to the bones and internal organs. The pregnancy rate for every group was 72 to 80% indicating that the calcium L-Threonate had no reproductive toxicity.

Six –Month (Long Term) Toxicity Study with Dogs

The study was conducted with hybrid dogs 0.5 to 1.5 years of age and weighing between 10 and 12kg.

The dogs were assigned as follows:-

- Control group;
- Low dose (1g / kg / day):
- Medium dose (2g / kg / day):
- High dose (3g / kg / day):

The test substance (Calcium L-Threonate) was administered orally each morning for 6 days a week with one rest day, for 24 continuous weeks. The calcium Threonate was mixed with about 200ml broth and fed to the animals in separate vessels before their normal feed

During the test period the following observations were recorded each day:

- general appearance
- psychomotility
- urine
- faeces
- appetite and quantity of food intake
- liquid intake

The following parameters were evaluated at regular intervals.

- Blood indices: RBC count, WBC count and classification, Hb, platelet count and coagulation time. Blood serum biochemistry (at 2, 4, 6 and 6.5 months).
- Cardiogram: 1, 11 and 111 lead, AVR, AVF and AVL lead (once every three months).
- Breath, blood pressure and body temperature (at 2, 4, 6 and 6.5 months).
- Body weight (every two weeks).
- Examination of urine and faeces before administration, 3 months and after administration period).

The results of these investigations showed no abnormality of any of the animals in the four groups. The higher doses appeared to have the effect of reducing the appetite of some of the animals. No abnormalities were found in the urine but the faeces of the dogs on the medium and high doses were thin and contained unabsorbed drug druff.

There was no significant difference in body weight changes in the animals in and between the four groups. Body average temperatures and blood pressures were within normal ranges throughout the study. No major fluctuations and no marked abnormalities were observed in any of the animals. Electrocardiogram readings were taken before administration, and at 3 and 6 months and at 6.5 months following completion of administration. No abnormalities were found in the cardiograms of the animals except that the animals in the high dose group showed a marked slowing of the heart rate to 93.3 ± 6.2 beats per minute at the 6 month check. Before administration the rates were 110.2 ± 20.0 / minute and two weeks after cessation of the administration they had returned to normal (110.6 ± 10.3).

The haematology indicated that there was no significant difference in the RBC and WBC counts and classification, Hb, platelet count and coagulation time of animals within and between males and females. No significant difference was observed in the blood serum biochemistry recorded before administration, at 2, 4 and 6 months and two weeks after administration.

Pathological Examinations

A pathological investigation was carried out on the dogs following the six month toxicity test. Two to three animals from each group were killed 24 hours after the last administration and the rest of the animals were killed two weeks after administration. A routine anatomical examination was carried out on all animals. The results of the histopathology on the organs and tissues were:

Cardiac muscle: The clear structure of endocardium, epicardium and cardiac muscle fibre were seen to have no histopathological changes.

Lung: Light interstitial focal fibroid hyperplasia , pulmonary alveoli wall incassation and inflammatory cell infiltration were found in very few animals.

Spleen: Clear splenic corpuscle and congestive splenic sinus were seen and no abnormal changes were observed.

Liver: Smooth hepatic capsula were seen and no histopathological changes of liver cells were observed. Focal round cell infiltration among lobi hepatis or in portal area were found in most of the animals (including control animals).

Kidney: A smooth, hard, light-yellow calculosis was found in right renal pelvis of an animal from the medium dosage group. Smooth nephrocapsula and clear structure of renal cortex and medulla was found. No inflammatory cell infiltration in the interstitial tissue of kidney and no histopathological changes of glomeruli or renal tubules cells were observed. No cast in the renal tubules.

G.I tract: No mucosal injury was found to either the stomach, duodenum, jejunum, ileum or colon. No exfoliation of epithelial cell was found.

Pancrease: No abnormal changes were observed.

Thyroid gland: Slight hyperplasia of thyroid gland was found in the high and medium dose groups. Flat epithelial cells of follicle turn into cubical or columnar epithelial cells. Neogenesis of small follicle and a quantitative decrease or absence of gelatinous substance was observed. Exfoliated cells were found in some follicles. No histopathological changes were observed in the low and medium dose groups. The changes observed in the high and medium dose immediately after cessation of the drug administration were reverse at the 2-week point.

Gonad: No abnormal changes in testicle, epididymis and ovary were observed.

Uterus: No abnormal changes were observed.

Urinary bladder: No abnormal changes were observed.

Thymus gland and lymph gland: No abnormal changes were observed.

Cerebra cortex, cerebellum and spinal cord: No abnormal changes were observed.

Marrow: No abnormal proportion of erythrocyte and leukocyte was found. A large mount of megakaryocyte and hematoblast were seen.

Skeletal muscles: No abnormal changes were observed.

Rib: The smooth periosteum and moderate dense of bone trabecula were observed. No abnormal changes of osteocyte, osteoblast and osteoclast were found. Over-calcification and fibrous hyperplasia of bone substance and bone trabecula was not observed

The results showed no abnormalities or histopathological changes specific to the administration groups, except for slight hyperplasia of the thyroid gland which was found in animals from the high and medium dose groups as indicated above. In these animals there was an apparent decrease or absence of gelatinous substance in follicles and cubical or columnar epithelial cells and exfoliated cells were also found in some follicles. It was considered that these observed changes may be attributed to the high dosage and long-term calcium intake. A high concentration of Ca^{2+} cannot only influence intestinal absorption of iodine but may also lead to an increase of Ca^{2+} in the epithelial cells of a follicle and thus reduce the secretion of thyroxin. No abnormal changes were observed in the thyroid in animals in the low dose and control groups.

Study on Acute Toxicity of Calcium L-Threonate

The acute oral toxicity of calcium L-Threonate was investigated using 20 Kunming mice and 20 Wistar rats, and the acute abdominal toxicity was investigated using 50 Kunming mice. Each group contained half males and half females.

For the oral toxicity study the mice and rats were fasted for 12 hours, with drinking water freely available. The mice were given calcium L-Threonate solution at a level of 2.5ml/100g body weight while the rats received calcium L-Threonate solution at a level of 2.0ml/100g body weight, both solutions being at a concentration of 0.8g/ml. Administration was twice daily at intervals of 6 hours. Both mice and rats were observed for 10 days with records made of any toxic reaction or change in body weight.

No change in behaviour was observed in the mice and rats during the study. No mortality occurred and no anomalies were detected upon autopsy at the end of the study. Weight change was observed, with the male mice and rats displaying a greater weight increase to that of the females. The maximum tolerated level of calcium L-Threonate was calculated as 40g/kg for mice and 32g/kg for rats.

For the abdominal toxicity study the mice were divided into five groups of 10 and received intraperitoneal injection of calcium L-Threonate solution (at a level of 2.0ml/100g body weight) at concentrations of 1500.00mg/kg, 1148.67mg/kg, 870.54mg/kg, 659.75mg/kg or 500.00mg/kg. The mice

were observed for any toxic reaction and mortality for 7 days. The Bliss method indicated that the LD₅₀ of calcium L-Threonate is 942.64mg/kg.

Investigation into Possible Pharmacological Effects

The possible pharmacological effects of calcium L-Threonate were investigated in two studies, one on neural systems of mice and the other on the cardiovascular and respiratory systems of anaesthetised dogs.

In the neural study the mice were given oral doses at 0.77, 1.54, 3.08 and 6.16g calcium Threonate / kg body weight (these were equivalent to 100, 200, 300 and 400mg / kg body weight of calcium). A control group was given a carboxymethyl cellulose solution (CMC) and as a positive contrast a sixth group received 10mg / kg of diazepam.

Voluntary behaviour of all mice was observed and recorded from 1 hour after oral administration. The mice were put into a recording box of multifunctional measuring instruments in a quiet room and observations made at 5 minute intervals.

Statistical analysis indicated that there were no significant differences between the voluntary behaviour times of the four calcium L-Threonate administration groups when compared to the CMC control group. The voluntary behaviour times of the diazepam group were less than that of the control group ($P < 0.01$).

After the observations on the voluntary behaviour of the mice there was an administration of 50mg / kg body weight of Nembutal and the Nembutal sleeping period was recorded.

A statistical analysis indicated that there were no significant differences in the Nembutal sleeping periods between the four calcium L-Threonate groups and the CMC control group.

The results are given in Table 10.

Table 10: Effect of Calcium L-Threonate on voluntary behaviour and Nembutal sleeping period in mice (X±SD)

Group	Dose	Number of animal	No. of voluntary behaviour (times)	Sleeping period (min)
Control group	0.5mg/10g	14	744±115	39±12
Calcium L-Threonate	6.160g/kg	12	752±108	39±13
Calcium L-Threonate	3.080g/kg	12	680±94	39±7
Calcium L-Threonate	1.540g/kg	12	696±82	38±14
Calcium L-Threonate	0.770g/kg	12	691±110	41±11
Diazepam group	1mg/kg	14	469±196**	73±39**

Compare with control group, **p<0.01

An associated study was carried out on healthy hybrid dogs to investigate the effects of calcium L-Threonate on heart rate, blood pressure and respiration. The dogs were given 30mg / kg Nembutal intravenously and a stomach tube implanted. Femoral blood pressure was measured by intubation whilst a respiration energy transducer placed in the animal's nostrils was used to record respiratory frequency and depth. An ECG was also conducted. All observations and recordings were made from 30 minutes after the operation. The calcium L-Threonate at dosage levels of 0.385, 0.77 and 1.54g / kg was administered by gastrogavage (the dosage levels were equivalent to 50, 100 and 200mg / kg body weight). The control animals received an equal volume of 0.5% carboxymethyl cellulose suspension. The test parameters were measured at 0.25, 0.5 and 1hr from the time of the administration and then at hourly intervals for 10 hours. No significant differences between the blood pressures and heart rates between the administration and control groups were observed (p >0.05). However, significant differences were observed at a few time points but these did not show any relationship to dosage. Similar results were observed in the respiratory frequency and depth. No significant effect was found on the ECG (p >0.05). The effects of calcium L-Threonate on the blood pressure and heart rate of conscious dogs was also studied. The dogs received either, 0.77g / kg, 1.54g / kg of calcium L-Threonate or a carboxymethyl cellulose solution. The dogs' carotid arteries had instrument receptors embedded in to measure the blood pressure whilst the dogs were conscious. Systolic and Diastolic blood pressure were measured by auscultation (mean of three measurements taken at 1 min intervals).

The test substances were administered through a stomach tube.

A statistical analysis of the results indicated that there were no significant differences between the blood pressures and heart rates between the control animals and those at the two administration dosages, either at the same time points or at different time points.

It was concluded that both the blood pressures and heart rates of conscious dogs were not affected by what could be considered a 'pharmacological' dose of calcium L-Threonate.

CONCLUSION

Human and animal studies indicate that calcium is absorbed from orally ingested calcium L-threonate. In animal studies, the bioavailability of calcium from calcium L-Threonate was comparable to or higher than that from other sources. Although, no data were provided on the metabolic fate of Threonate, its noted that Threonate is a normal constituent of the body. For example, it has been identified in human plasma and urine. Threonate typically arises from the catabolism of ascorbic acid. Acute oral toxicity studies revealed that calcium L-Threonate is of low toxicity, with no adverse effects observed at doses as high as 40 g/kg bw in mice or 32 g/kg bw in rats. In sub-chronic studies with calcium L-Threonate, the Panel identified a NOAEL of 4 g/kg bw/day in the rat with regard to its effect on blood coagulation time and accretion of the thyroid gland, and of 1 g calcium L-Threonate/kg bw/day in the dog with regard to hyperplasia of the thyroid gland. The Panel noticed that the effects on blood coagulation time and the thyroid gland were reversible and that a mild accretion in the thyroid gland in rats was limited to males only. Its further noticed that these effects were likely to be attributed to the high dosage of calcium administered over a long period. A high concentration of calcium ions can result in accelerated blood coagulation. It can influence intestinal absorption of iodine and reduce/suppress the secretion of thyroxin by the thyroid gland. The NOAELs are equivalent to 516 mg calcium/kg bw/day and 3484 mg L-Threonate/kg bw/day in the rat and to 129 mg calcium/kg bw/day and 871 mg L-Threonate/kg bw/day in the dog. Studies using different test systems *in vitro* and *in vivo* indicated that calcium L-Threonate was not genotoxic. Although no carcinogenicity studies were available, its considered that such studies were not needed given that L-Threonate is an endogenous compound in the body and that calcium L-Threonate did not show any genotoxic potential. Reproductive and developmental toxicity studies in mice indicated that calcium L-Threonate in doses up to 6 g/kg bw/day had no adverse effect on the fertility and on the developing foetus, nor did it

cause maternal toxicity. The SCF has established a tolerable upper intake level of 2500 mg/day for calcium from all sources for adults. The petitioner proposed use levels of 2 - 4 tablets/person/day of calcium L-Threonate with each tablet providing 100 mg calcium and 675 mg Threonate. The exposure

to calcium through the use and at the use levels of calcium L-Threonate proposed by the petitioner, may lead to exposures of 200-400 mg calcium/person/day, which would not represent a safety concern. Data on dietary intakes of L-Threonate were not available. L-Threonate may occur in certain foods. For instance, L-Threonate is a breakdown product of ascorbic acid during food preparation. Exposure to L-Threonate at the uses and use levels indicated by the petitioner is estimated to amount to 1350 - 2700 mg L-Threonate per person per day corresponding to 22.5 - 45 mg/kg bw/day for a 60 kg person. The margin of safety between the estimated human exposure to L-Threonate and the amount of L-Threonate equivalent to the NOAELs for calcium L-Threonate, as demonstrated in sub-chronic toxicity studies in dogs and rats, is 39 - 19 for the dog and 155-77 for the rat. The Panel considers this margin of safety to be sufficiently large given that Threonate is an endogenous compound in the body, and that the NOAELs in the dog and rat studies were identified for effects attributable not to L-Threonate but to the calcium dosages.

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