Section A6.4 Repeated dose toxicity

Annex Point IIA6.3 / 6.4 / 6.5

13 weeks - rat

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1989. Subchronic Oral Toxicity study of Calcium lactate in F344 Rats Bulletin of the National Institute of Hygienic Sciences, Tokyo (Eisei Shikenjo Hokuku) Vol. 107: pp 78-83.	
1.2	**Data protection**	No	
1.2.1	Data owner	Literature publication	
1.2.2	Companies with letter of access	No	
1.2.3	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Not applicable, literature publication	
2.2	**GLP**	Not applicable, literature publication	
2.3	**Deviations**	Not applicable, literature publication	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Calcium lactate pentahydrate ($C_6H_{10}CaO_6 \cdot 5H_2O$ 308.30) In the current study calcium lactate dissolved in water was tested. As it is administered dissolved in water, the results of this study can be used for lactic acid.	
3.1.1	Lot/Batch number	Sample obtained from Musashino Chemical Inst. Ltd (Tokyo, Japan)	
3.1.2	Specification	Deviating from specification given in section 2 as follows Product contains calcium lactate at 97.0% to 101% when calculated as a dried product. Product was colourless and clear, pH 6.0-8.0; heavy metals (Pb) 20 µg/g maximum; alkaline metals and magnesium 1% maximum, arsenic < 4 µg/g maximum.	
3.1.2.1	**Description**	Odourless white powder or granules	
3.1.2.2	**Purity**	97.0 – 101.0 % when calculated as dried product	
3.1.2.3	**Stability**	Not reported	
3.2	**Test Animals**		
3.2.1	Species	Rat	
3.2.2	Strain	Male rats: SPF Female rats: F344/DuCrj	X
3.2.3	Source	Charles River Laboratories Japan, Inc.	

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3.2.4	Sex	Male and female	
3.2.5	Age/weight at study initiation	Five weeks old	X
3.2.6	Number of animals per group	Experiment I: 5 males and 5 females/group Experiment II: 5 male and 5 females/group Experiment III: 10 male/group	
3.2.7	Control animals	Yes	
3.3	**Administration/ Exposure**	Oral	
3.3.1	Duration of treatment	Experiment I: 13 weeks Experiment II: 20 weeks Experiment III: 8 weeks	
3.3.2	Frequency of exposure	Daily, ad libitum	
3.3.3	Postexposure period	No post-exposure period	
3.3.4	**Oral**		
3.3.4.1	**Type**	Experiment I: in drinking water Experiment II: in food Experiment III: no lactate, comparison of CRF-1 solid diet (used in experiment I) and B-blend power diet (used in experiment II) (both supplied by Oriental Yeast Co., Ltd.)	
3.3.4.2	**Concentration**	Experiment I: 0, 0.3, 0.6, 1.25, 2.5, and 5 % Experiment II: 0, 5, 10, 20, 30 % Experiment III: no lactate, comparison of diets	
3.3.4.3	**Vehicle**	Experiment I: Ion-exchanged water Experiment II: in standard blend of purified diet (B-blend powder diet, Oriental Yeast Co., Ltd.) Experiment III: no lactate, comparison of diets	
3.3.4.4	**Concentration in vehicle**	Experiment I: 0, 0.3, 0.6, 1.25, 2.5, and 5 % Experiment II: 0, 5, 10, 20, 30 % Experiment III: no lactate, comparison of diets	
3.3.4.5	**Total volume applied**	Ad libitum	
3.3.4.6	**Controls**	vehicle	
3.4	**Examinations**		
3.4.1	Observations	Weekly	
3.4.1.1	**Clinical signs**	Yes	

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3.4.1.2	**Mortality**	Yes
3.4.2	Body weight	Yes, weekly
3.4.3	Food consumption	Yes
3.4.4	Water consumption	Yes
3.4.5	Ophthalmoscopic examination	No
3.4.6	Haematology	Yes, number of animals: surviving animals time points: end of study Parameters: erythrocyte, leucocyte, haemoglobin, haematocrit, MCV.
3.4.7	Clinical Chemisty	Yes, number of animals: surviving animals time points: end of study Parameters: GOT, GPT, LDH, AIP, TTT, total billirubin., total cholesterol, TG, β-Lipo-protease, total protein, A/G, BUN, Creatinine, Uric acid, ZTT, γ-GTP, calcium,
3.4.8	Urinalysis	Yes, in experiment II number of animals: not specified time points: in the 18th week of administration Parameters: volume, calcium
3.5	**Sacrifice and pathology**	
3.5.1	Organ Weights	Yes, organs (only reported when significant changes were observed): heart, brain.
3.5.2	Gross and histopathology	Yes, all dose groups/ high dose group and controls, other dose groups only if effects organs(only reported when abnormalities were observed): lymph nodes, Harderian gland, lungs, heart, grandular stomach, liver, spleen, kidney, testis, prostate gland, bone marrow.
3.5.3	Other examinations	Calcium deposit on the urinary tubule
3.5.4	Statistics	Not reported
3.6	**Further remarks**	Not applicable

4 RESULTS AND DISCUSSION

4.1	**Observations**	
4.1.1	Clinical signs	Experiment I: no abnormalities Experiment II: no abnormalities reported Experiment III: no abnormalities reported
4.1.2	Mortality	No mortality observed in all three experiments

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4.2	**Body weight gain**	Experiment I: Male rats in the 1.25 and 5% groups showed slight inhibition of weight gain, but this inhibition stayed within 10% of the control group. No significant difference was seen in the female rats between groups. Experiment II: Significant inhibition of body weight gain in both males and females in the highest dose group (30%), and in the males of the 20% group. Experiment III: Not reported	
4.3	**Food consumption and compound intake**	Experiment I: Average intake of drinking water was 75% of the control group in the highest dose (5%) and 88% in the 2.5% group. Total intake of test substance was calculated from the intake of drinking water. Experiment II: Not reported Experiment III: Not reported	X
4.4	**Ophtalmoscopic examination**	Not applicable	
4.5	**Blood analysis**		
4.5.1	Haematology	Experiment I: Variations observed in male rats could not be correlated with doses Experiment II: No correlation with doses was found Experiment III: Not reported	
4.5.2	Clinical chemistry	Experiment I: Slight increases in BUN and creatinine levels were observed in female rats in the 0.6, 1.25, 2.5, and 5% groups. Increases in LDH levels were observed in the 0.6, 1.25, 2.5, and 5% groups, and increases in GOT levels in the 1.25, 2.5, and 5% groups. In the females from all of those groups, slight correlation with doses was noted. Experiment II: Not reported Experiment III: Not reported	X
4.5.3	Urinalysis	Experiment I: Not reported. Experiment II: The urinary output of male rats was approximately 6 mL in two highest dose groups (20 en 30%), which was twice the amount of the control group (3 mL). In females, the urinary output was almost 5 mL in all groups. Calcium concentrations significantly increased with higher doses in both males and females, and correlated with the doses. Experiment III: Not reported	
4.6	**Sacrifice and pathology**		
4.6.1	Organ weights	Heart weight was significantly lower in the 5% group of male rats, compared to the control group. Based on the body weight ratios, the brain weight was significantly lower in the 1.25% group of males.	

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4.6.2	Gross and histopathology	Experiment I: No severe toxicological findings were observed in any of the treated groups. Experiment II: Nephrocalcinosis was observed in all groups, including the control group, and an inverse dose-effect relation was observed with regard to the degree of its development. Experiment III: Nephrocalcinosis was found only in the group fed B-blend power diet (used in experiment II)	
4.7	**Other**	Not reported	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	Experiment I: Six groups of rats (5 males and 5 females) were exposed to calcium lactate dissolved in the drinking water (ad libitum) at concentrations of 0, 0.3, 0.6, 1.25, 2.5, and 5% for 13 weeks. Experiment II: Five groups of rats (5 males and 5 females) were exposed to calcium lactate in the diet (ad libitum) at concentrations of 0, 5, 10, 20, or 30% calcium lactate. Experiment III: Two groups of ten male rats were each given a different diet, one group the diet used in experiment I (CRF-1 solid diet), and the other group the diet used in experiment II (β-blend powder diet).	
5.2	**Results and discussion**	Experiment I: No mortality was observed. Hematological and hematobiochemical studies showed slight increases in BUN, creatinine, LDH, and GOT in females, which could be correlated with the doses. However, pathohistologically, there were no findings supporting possible nephrotoxicity and/or hepatotoxicity.	X
		Experiment II: No mortality was observed. In the highest dose group, body weight gain was strongly reduced compared to the control group. Histological examination revealed nephrocalcinosis in all groups, including the control group, and an inverse dose-effect relation was observed with regard to the degree of its development.	
		Experiment III: Nephrocalcinosis was observed only in the group fed the diet used in experiment II (β-blend powder diet). It was concluded that the β-blend powder diet causes nephrocalcinosis due to a low Ca/P ratio. Exposure to calcium lactate in experiment II causes increases in the Ca/P ratio, resulting in increased nephrocalcinosis. In experiment I, the CRF-I diet was given, which contains a high Ca/P ratio, which explains why in experiment I no nephrocalcinosis was found. Nephrocalcinosis can therefore not be attributed to the exposure to calcium lactate.	X
5.3	**Conclusion**		
5.3.1	LO(A)EL	Not reported. Based on increased creatinine levels in blood, the LOAEL can be set at 0.6%. As no exact doses are mentioned in the publication, no exact LOAEL (mg/kg) can be set.	X
5.3.2	NO(A)EL	Not reported. Based on increased creatinine levels in blood, the NOAEL can be set at 0.3%. As no exact doses are mentioned in the publication, no exact NOAEL (mg/kg) can be set.	X

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5.3.3	Other	This study was used to determine the optimal dose for a long-term toxicity/carcinogenicity study. Based on the values obtained from Experiment I, 5 and 2.5% were used in this study (see A6.5-01 and A6.7-01)	
5.3.4	Reliability	2	
5.3.5	Deficiencies	Yes, study is not performed according to current guidelines. As it is a literature publication, the reporting is concise and raw data are missing. However, the study has been performed well and can be used for the purpose of this dossier. As calcium lactate was used, effects of calcium should also be taken into account.	X

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	2008/06/30
Materials and Methods	3.2.2 F344/DuCrj (both sexes) 3.2.5 6 weeks at study begin
Results and discussion	The applicant's version is acceptable with the following amendment: 4.3 Experiment II: Food consumption in the high dose group was 65 % and 57 % of the food intake of control group for males and females, respectively. 4.5.2 The increases in clinical chemistry parameters were slight and not dose-dependent, except for LDH (see CA-table 1). 5.2 Hematological and hematobiochemical studies showed slight increases in BUN, creatinine, LDH, and GOT in females, which could not be correlated with the doses except for LDH. Experiment III: Exposure to calcium lactate in experiment II causes increases in the Ca/P ratio, resulting in decreased nephrocalcinosis.
Conclusion	LO(A)EL: 30 % lactic acid in food, ~12 g/kg bw/d NO(A)EL: 20 % lactic acid in food, ~ 8.5 g/kg bw/d
Reliability	2
Acceptability	Acceptable with restrictions
Remarks	The results of this study can used as a very rough approximation for a NOAEL for L(+) lactic acid because the effects observed (decrease in food consumption and body weight gain) might be due to high calcium intake, palatability problems and/or malabsorption due to local gastrointestinal irritation (provoked by calcium or lactate). Thus, in the view of the RMS, the study seems to be inadequate to use the obtained NOAEL for derivation of reference values.
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*

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Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 1. Serum biochemistry and hematology results (Exptl. I, male)

Dose (%)	Control	0.3	0.6	1.25	2.5	5
Effective No.	10	10	10	10	10	10
GOT(KU)	94.2±8.9	87.4±10.6	85.6±11.2	167.2±58.3*	83.4±6.7	96.2±4.6
GPT(KU)	28.8±4.3	31.2±2.6	28.6±3.0	27.4±2.9	30.0±3.8	26.2±3.6
LDH(WU)	102±313	512±349*	684±445	847±623	496±211*	1121±166
AlP(KAU)	12.1±0.9	12.1±0.8	12.8±0.6	12.7±0.8	12.6±0.7	13.1±0.6
TTT(SHU)	0.58±0.23	0.64±0.15	0.82±0.21	0.70±0.35	0.84±0.23	0.90±0.27
Total-bil.(mg/dl)	0.44±0.11	0.50±0.15	0.42±0.17	0.48±0.14	0.42±0.14	0.42±0.17
Total-cho.(mg/dl)	56.2±2.2	55.6±3.1	54.8±2.2	58.6±2.1	55.0±4.4	56.0±4.4
TG(mg/dl)	164±29	167±19	156±16	196±29	154±54	159±23
β-Lipo-pro.(mg/dl)	132±24	137±19	124±15	161±29	114±43	137±23
Total-pro.(g/dl)	7.20±0.14	7.30±0.07	7.08±0.08	7.12±0.13	6.96±0.23	6.92±0.13
A/G	1.54±0.05	1.50±0.07	1.54±0.11	1.50±0.07	1.58±0.08	1.58±0.10
BUN(mg/dl)	19.0±1.2	19.2±0.8	19.0±1.0	16.4±2.7	20.0±1.2	21.4±0.9*
Creat.(mg/dl)	0.56±0.05	0.54±0.05	0.48±0.04*	0.56±0.05	0.54±0.05	0.56±0.05
Uric acid(mg/dl)	1.76±0.59	1.54±0.15*	1.52±0.16*	1.72±0.17*	1.84±0.88	1.94±0.25
ZTT(KU)	0.56±0.08	0.50±0	0.56±0.05	0.66±0.11	0.50±0.10	0.64±0.05
γ-GTP(mu/ml)	1.60±0.54	1.60±0.54	1.60±0.54	1.40±0.54	1.40±0.54	2.40±0.89
Ca(mg/dl)	9.90±0.97	11.06±0.37*	10.80±0.07	11.16±0.82	11.54±0.52*	10.74±0.49
Erythrocyte($\times10^4$/mm^3)	951±31	975±19	951±31	942±14	943±59	910±76
Leucocyte($\times10^3$/mm^3)	95±12	74±15*	47±12**	51±7**	58±5**	60±3**
Hb.(g/dl)	175±3	175±7	164±5**	166±4**	178±9	198±38
Ht.(%)	52.0±1.0	50.8±0.4*	50.6±0.5*	50.8±0.4*	51.3±1.3	52.7±1.3
MCV(μ^3)	496±7	496±8	470±18*	478±9*	484±21	481±31

* : $p<0.05$ ** : $p<0.01$ Mean±SD

Table 2. Serum biochemistry and hematology results (Exptl. I, female)

Dose (%)	Control	0.3	0.6	1.25	2.5	5
Effective No.	10	10	10	10	10	10
GOT(KU)	74.0±6.7	77.0±7.9	74.4±3.4	85.0±8.3*	88.4±6.2**	90.0±6.0**
GPT(KU)	17.8±2.7	21.6±5.0	21.2±2.6	18.6±5.2	23.4±4.7	24.0±2.9**
LDH(WU)	267±179	473±227	586±221*	880±271**	889±403*	712±232**
AlP(KAU)	8.0±0.8	9.1±1.0	8.0±1.0	8.5±1.4	8.0±0.6	8.3±0.4
TTT(SHU)	0.74±0.13	0.88±0.42*	0.90±0.12	0.86±0.32	0.82±0.23	1.12±0.23*
Total-bil.(mg/dl)	0.58±0.08	0.46±0.18	0.54±0.19	0.62±0.14	0.42±0.13*	0.62±0.15
Total-cho.(mg/dl)	94.4±6.1	92.6±3.8	92.8±4.7	88.4±4.0	90.2±8.0	86.2±4.7*
TG(mg/dl)	114±18	120±35	116±33	110±24	119±14	147±29
β-Lipo-pro.(mg/dl)	96±20	105±36	95±32	91±23	108±20	131±31
Total-pro.(g/dl)	7.02±0.10	7.04±0.16	6.96±0.16	7.16±0.23	7.10±0.12	7.08±0.26
A/G	1.66±0.05	1.64±0.08	1.64±0.08	1.60±0.10	1.62±0.13	1.54±0.23*
BUN(mg/dl)	18.2±0.8	18.6±0.9	18.6±2.1	21.2±1.5**	22.0±1.4**	21.0±4.0
Creat.(mg/dl)	0.46±0.05	0.50±0.10	0.54±0.05*	0.54±0.05*	0.56±0.05*	0.66±0.05**
Uric acid(mg/dl)	1.96±0.15	1.96±0.28	2.16±0.19	2.16±0.08*	2.26±0.40	2.34±0.28*
ZTT(KU)	0.58±0.13	0.64±0.11	0.74±0.18	0.68±0.10	0.70±0.24	0.88±0.13**
γ-GTP(mu/ml)	1.60±0.54	2.20±0.44	2.00±0.70	2.20±0.44	1.80±0.44	2.00±0.70
Ca(mg/dl)	10.48±0.76	10.38±0.81	10.88±0.66	10.06±0.52	10.06±0.50	10.24±0.23*
Erythrocyte($\times10^4$/mm^3)	912±24	894±33	907±17	905±12	897±18	916±46
Leukocyte($\times10^3$/mm^3)	50±8	58±8	41±6	59±9	49±9	74±10**
Hb.(g/dl)	154±5	161±9	162±4	164±3	161±4	164±11
Ht.(%)	53.6±0.5	54.0±0.7	53.6±0.5	53.6±0.5	53.8±0.5	53.6±0.5
MCV(μ^3)	488±12	482±16	486±8	485±7	482±9	491±24

* : $p<0.05$ ** : $p<0.01$ Mean±SD