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# Toxicologic evaluation of potassium polyaspartate (A-5D K/SD): Genotoxicity and subchronic toxicity

C. Galbusera [a, *], C. Casalegno [a], S. Marroncelli [a], G. Triulzi [b], J. Santos [c], E. Corsini [d], P. Restani [d]

[a] ChemService srl Controlli e Ricerche, Via Fratelli Beltrami 15, 20026, Novate Milanese, MI, Italy
[b] Esseco S.r.l., Via S. Cassiano 99, 28069 Trecate, NO, Italy
[c] Enartis USA, 7795 Bell Rd, Windsor, CA 95492, United States
[d] Dept. Pharmacological and Biomolecular Sciences, University of Milan, via Balzaretti 9, 20133 Milano, Italy

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ABSTRACT

Potassium polyaspartate (A-5D K/SD) is proposed for use as a stabiliser in wine, with a maximum use level of 300 mg/L and typical levels in the range of 100–200 mg/L. Potassium polyaspartate (A-5D K/SD) tested negative in a bacterial reverse mutation assay performed in accordance with OECD TG 471 and in an *in vitro* mammalian cell micronucleus test performed in accordance with OECD TG 487. From a 90-day oral toxicity study in male and female Wistar rats performed in accordance with OECD TG 408, a no observed adverse effect level (NOAEL) was set at 1000 mg/kg bw per day, the highest dose tested. In its opinion adopted on 9 March 2016, the EFSA-ANS Panel (European Food Safety Authority - Panel on Food Additives and Nutrient Sources added to Food), considering these data, concluded that "there was no safety concern from the proposed use and use levels of potassium polyaspartate (A-5D K/SD)".



## 1. Introduction

Potassium polyaspartate (A-5D K/SD) is the potassium salt of polyaspartic acid, synthesized from L-aspartic acid and potassium hydroxide. The chemical structure is shown in Fig. 1.

Studies have verified that different types of polyaspartates have stabilizing properties similar to those of the metatartaric acid (MTA), but are much more stable over time (Bosso et al., 2015). Potassium polyaspartate (A-5D K/SD) has been authorized for experimental trials of tartaric stabilization in white, rosé and red wines, in accordance with Art 4 of Commission Regulation (EC) No 606/2009, in Italy (Italian Ministry of Agricultural, 2014) and in Spain (Department of Agriculture, 2014). The same request was submitted to the relevant competent authority of France on 22 December 2014 (General Directorate for Competition Policy). These authorizations have been granted/requested after the submission of physico-chemical, toxicokinetic, immunological and (geno)toxicological data available for A-5D K/SD.

EFSA (European Food Safety Authority) evaluated the safety of potassium polyaspartate as a food additive and in its opinion of 9 March 2016 (EFSA Journal, 2016) concluded that there was no safety concern from the proposed use in wine at a maximum use level of 300 mg/L and typical levels in the range of 100–200 mg/L.

Draft regulation amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council and the Annex to Commission Regulation (EU) No 231/2012 as regards potassium polyaspartate has been published (http://ec.europa.eu/transparency/regcomitology/index.cfm?do=search.documentdetail&Dos_ID=14196&ds_id=50363&version=2&page=1&AttLang=en) and it will enter into force on the twentieth day following that of its publication

*Abbreviations:* OJEU, Official Journal of the European Union; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; APTT, Activated Partial Thromboplastin Time; AST, aspartate aminotransferase; BNCs, micronucleated binucleated cells; bw, body weight; CBPI, Cytokinesis Block Proliferation Index; CPM, cyclophosphamide; EFSA, European Food Safety Authority; F, female; GLP, Good Laboratory Practice; Hb, hemoglobin; IARC, Agency for Research on Cancer; M, males; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; MCV, Mean Corpuscular Volume; mg/kg, milligrams per kilogram; MMC, mitomycin C; MN, micronucleus or micronuclei; MTA, metatartaric acid; NOAEL, No Observed Adverse Effect Level; NTP, National Toxicology Program; OECD, Organization for Economic Cooperation and Development; OSHA, Occupational Safety & Health Administration; PCV, Packed Cell Volume; PT, Prothrombin Time; RBC, red blood cell count; S9, metabolic activation system; SD, Standard Deviation; T3, Triiodothyronine; T4, thyroxine; TSH, Thyroid Stimulating Hormone; VBC, vinblastine; WBC, total white blood cell count.

* Corresponding author.
*E-mail address:* c.galbusera@chemservice.it (C. Galbusera).

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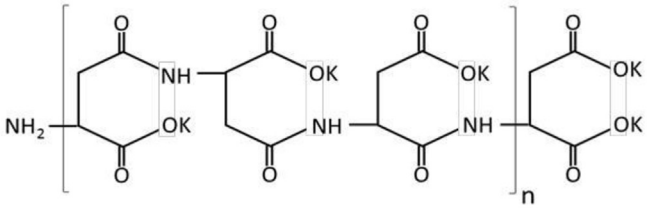


$[C_4H_4NO_3K]_n$

Number average molecular weight = 1,100 g/mol

Weight average molecular weight = 5,300 g/mol

**Fig. 1.** Chemical structure of potassium polyaspartate (A-5D K/SD).

in the Official Journal of the European Union (OJEU).

Polyaspartic acid is not listed by the US National Toxicology Program (NTP) or the International Agency for Research on Cancer (IARC), nor it is regulated as a carcinogen by the US Occupational Safety & Health Administration (OSHA). Authorizations and evaluations are available for the sodium salt of polyaspartic acid (CAS 34345-47-6), the polymer containing sodium instead of potassium. The sodium salt of polyaspartic acid is authorized for use in USA (Environmental Assessment LANXESS Deutschland GmbH, Food Contact Notification, 2007) (Environmental Assessment Food Contact Notification, NanoChem Solutions, 2013) and Australia (Summary Report "2-Butenedioic acid (2Z)-, ammonium salt, homopolymer, hydrolysed, sodium salts" Reference No: NA/932) as a food contact substance, as a dispersant for fillers and an anti-scale additive in sugar processing; and as a water treatment agent used as a scale inhibitor in cooling tower and boiler water applications.

Potassium polyaspartate (A-5DK SD) is produced from L-aspartic acid according to the reaction reported in Fig. 2. The thermic process transforms the aspartic acid in polysuccinimide that is insoluble. Polysuccinimide is then treated with potassium hydroxide under controlled conditions, thus allowing the opening of the ring and polymerisation of the units. Through this process, a 40% solution of potassium polyaspartate at pH 8.3 is obtained.

The last step of the production of the preparation of potassium polyaspartate (A-5D K/SD) is the spray drying phase, which results in a light tan powder at 92–95% dry matter.

The studies performed in the present study were conducted to examine the safety of potassium polyaspartate (A-5DK SD). Potassium polyaspartate (A-5DK SD) was evaluated in a Good Laboratory Practice (GLP) test battery compliant with EFSA and OECD guidance on genotoxicity testing (EFSA, 2008; OECD, 1997a; OECD 2008a; OECD 2008b). Specifically, potassium polyaspartate (A-5DK SD) was evaluated in a bacterial reverse mutation assay (OECD 471,1997) using Salmonella tester strains. In addition, an *in vitro* mammalian cell micronucleus MN (OECD 487, 2008a) was conducted in cultured human lymphocytes. Moreover, two toxicity studies were conducted, a 14-day range-finding study performed to collect information of target organs and to appropriate dosing, and a 90-day subchronic toxicity study in accordance with the OECD 408 (1998), modified to include assessment of additional parameters to allow for the identification of chemicals with the potential to cause neurotoxic, immunological or reproductive organ effects or endocrine-mediated effects.

This research has received funding from the European Community's Seventh Framework Programme and has been carried out within the Project "*Use of biopolymers for sustainable stabilization of quality wines*" – *Stabiwine*.

## 2. Materials and methods

The test material and methods employed are described for each individual study. In all studies, the stated concentrations or doses reflect the amount of potassium polyaspartate (A-5D K/SD) administered, and appropriate control groups were employed as necessary.

### 2.1. Test material

Potassium polyaspartate (A-5D K/SD) is potassium salt of polyaspartic acid (CAS: 64723-18-8) and its nominal purity is 98% w/w on dry matter and the overall impurities are lower than 2%.

The specifications (see Table 1) have been defined through the analysis of the content of active substance and impurities of five typical production batches of potassium polyaspartate (A-5D K/SD)

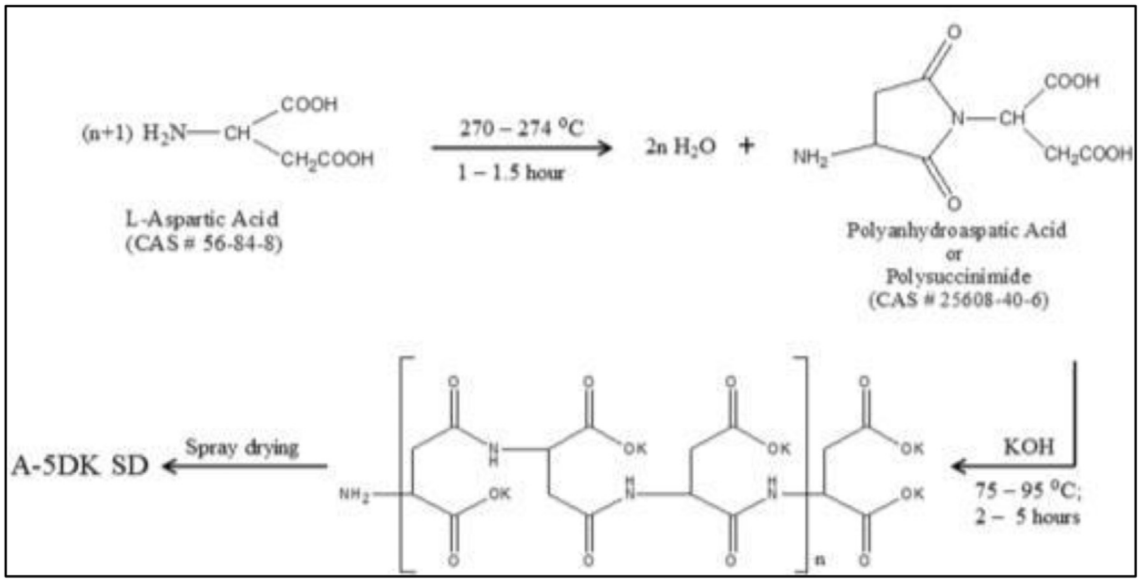


**Fig. 2.** Manufacturing process for potassium polyaspartate (A-5DK SD).

**Table 1**
Specifications for potassium polyaspartate (A-5D K/SD).

| Parameter | Specification | |
|---|---|---|
| Description: | Light brown powder without odour | |
| Identification: | | |
| pH | 7.5–8.5 (40% aqueous solution) | |
| Solubility | Water | >1000 g/L |
| | Xylene | <5.0 g/L |
| | Dichloromethane | <5.0 g/L |
| | Methanol | <5.0 g/L |
| | Acetone | <5.0 g/L |
| | Ethylacetate | <5.0 g/L |
| | *n*-Heptane | <5.0 g/L |
| Purity: | | |
| Nominal purity | Not less than 98.0% w/w on dry matter | |
| Degree of substitutions | Not less than 91.5% w/w on dry matter | |
| KOH | Not more than 2.0% w/w on dry matter | |
| Aspartic acid | Not more than 1.0% w/w | |
| Other significant impurities | Not more than 0.1% w/w | |

from the production plant. The batches were selected to cover a manufacturing period ranging from 2012 to 2015. Information on the batches tested is reported in Table 2.

The batch KHKS-040412 has been tested in the genotoxicity and subchronic studies.

### 2.2. Genotoxicity studies

All genotoxicity studies were conducted in accordance with OECD guidelines and GLP compliance (Good Laboratory Practice ENV/MC/CHEM (98)17).

#### 2.2.1. Bacterial reverse mutation assay

The procedures used in this study were in accordance with OECD Guideline 471 (OECD, 1997). Potassium polyaspartate (A-5D K/SD) was evaluated in an Ames/*Salmonella* pre-incubation assay to determine its ability to induce reverse mutation at selected histidine loci in five tester strains of *Salmonella typhimurium* (TA1535, TA97a, TA98, TA100 and TA102) in the presence and absence of a metabolic activation system (S9). Liver S9, induced in Wistar rats by phenobarbitone with β - naphthoflavone, was used for this purpose.

Based upon the preliminary tests conducted to assess the solubility/precipitation and cytotoxicity of A-5D K/SD, the tester strains were exposed to the test substance in triplicate cultures at the doses of 5000 μg, 1500 μg, 500 μg, 150 μg and 50 μg/plate both in the presence and absence of a metabolic activation system (S9). Analytical grade water was used as a vehicle. The exposed bacteria were plated onto minimal glucose agar medium supplemented with L-histidine. The plates were incubated at 37 °C for 68–69 h after which the histidine revertant colonies were counted and their frequency was compared with that in the vehicle control group. Strain specific positive controls also tested without metabolic activation were sodium azide (TA1535; 2 μg/plate), ICR 191 (TA97a; 1 μg/plate), 4-nitroquinoline-N-oxide (TA98; 0.5 μg/plate), 3-methylmethane sulphonate (TA100 and TA102; 1 μg/plate). For the experiment with the metabolic activation system, 2-aminoanthracene (TA1535; 10 μg/plate), 2-aminofluorene (TA97a, TA98 and TA100; 20 μg/plate), Danthron (TA102; 30 μg/plate) were used as positive controls. In order to confirm the reproducibility of the results, the entire study was carried out twice as experiment No.1 and experiment No.2.

#### 2.2.2. In vitro mammalian cell micronucleus assay

The methodology used in this study was based on OECD Test Guideline 487 (OECD, 2008a). The objective of the *in vitro* micronucleus assay was to evaluate the test substance, potassium polyaspartate (A-5D K/SD), for its ability to induce the formation of small membrane-bound DNA fragments such as micronuclei in the cytoplasm of the interphase cell. The experiment has been carried out in accordance with ethical principles provided by "Indian Council of Medical Research" (Ministry of Health, Government of India) Ethical Guidelines for Research (which are in lines with Code of Ethics of World Medical Association's Declaration of Helsinki - DoH, 1998).

An appropriate volume of whole blood from one healthy young-adult, non-smoking male volunteer was collected from the peripheral circulation into heparinized tubes. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 ml of pooled heparinized blood into 9.6 mL of medium (RPMI 1640-10 medium and phytohaemagglutinin-M) and were incubated at 37 °C for 48 ± 1 h. After incubation, each of the cultures tubes were centrifuged and the supernatant culture medium was removed and the cells were re-suspended in fresh culture medium to achieve a final pre-treatment volume of 10 mL. Cultures of human peripheral blood lymphocytes were exposed to potassium polyaspartate (A-5D K/SD) dissolved in analytical grade water at concentrations of 5000, 1500 and 500 μg/mL (concentrations based upon preliminary solubility/precipitation and cytotoxicity studies) with and without the metabolic activation system (i.e. rat liver S9). Duplicate cultures were used at each concentration. In experiments No. 1 and No. 2, cells were exposed to the test substance for 3 and 20 h, respectively, without the supplementary metabolic activation system. In experiment No. 3, conducted with the supplementary metabolic activation system, the cells were exposed for 3 h to the test substance, 48 h after culture initiation. Cytochalasin B (actin polymerisation inhibitor) was added at 68 h, whereas cell harvesting was performed 96 h after culture initiation. Positive, negative and vehicle controls, both with and without metabolic activation, were tested concurrently with the test substance. Analytical grade water was used as a vehicle. Mitomycin C (MMC) and vinblastine (VBC), known micronucleus forming agents, were employed as positive controls, at concentrations of 0.8 and 0.08 μg/mL respectively, for the experiments without the metabolic activation system, whereas cyclophosphamide (CPM) was employed at a concentration of 6.25 μg/mL for the experiment with the metabolic activation system. Each culture was harvested and slide preparations were made and stained with 5% Giemsa (OECD 487, 2008a). The cytotoxicity was determined from the number of cell cycles per cell during the period of exposure to cytochalasin B (CBPI, Cytokinesis-Block Proliferation Index) derived from at least 500 cells per culture (1000 cells per test concentration). Two thousand binucleated cells with well spread cytoplasm were evaluated microscopically for the presence of micronuclei, if any.

**Table 2**
Results of analysis of five production batches of potassium polyaspartate (A-5D K/SD).

| Batch No. | KHKS-040412 | KHKS-072512–1 | KHKS-070214–1 | KHKS-060414–1 | KDK-110515–1 |
|---|---|---|---|---|---|
| Nominal purity (% w/w) | 99 | 101 | 100 | 100 | 101 |
| Degree of substitution (% w/w) | 94 | 96 | 94 | 96 | 98 |
| Aspartic acid (% w/w) | 0.4 | 0.4 | 0.5 | 0.5 | 0.4 |

### 2.3. Oral toxicity studies

Two oral toxicity studies were performed in rats:

- a 14-day range-finding study performed to collect information of target organs and to select appropriate doses for a 90-day study;
- a 90-day subchronic toxicity study (OECD TG 408, 1998), modified to include assessment of additional parameters described in the more recent guideline on repeated-dose 28-day oral toxicity study in rodents (OECD TG 407, 2008b). This approach allows the identification of chemicals with the potential to cause neurotoxic, immunological or reproductive organ effects or endocrine-mediated effects, as recommended in EFSA Guidance for submission for food additive evaluations (EFSA, 2012).

#### 2.3.1. Test material and animals

Potassium polyaspartate (A-5D K/SD) was administered by oral gavage in the 14-day toxicity study and a subsequent 90-day toxicity study in rats. Concurrent vehicle control group receiving analytical grade water. Male and female rat (*Rattus norvegicus*) were housed in stainless steel cages in temperature-controlled and humidity-monitored quarters. Before starting the experiment, the animals were acclimatized for a minimum period of seven days. One day before treatment, animals were weighed and arranged according to their weight range. These body weight stratified rats were distributed to all experimental and control groups using random number table, so that body weight variation of animals selected for study did not exceed ±20% of the mean body weight of each sex.

#### 2.3.2. Experimental designs

The studies were conducted in accordance with OECD guidelines for the Testing of Chemicals and Food Ingredients, Section 4 (Parts 407 and 408): Health Effects (2008, 1987). The studies were performed under the conditions recommended by the "Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Guidelines for Laboratory Animal Facility" (CPCSEA, 1998) and "Guide for the care and use of laboratory animals" (NRC, 2011), US publication by National Research Council Institute of Laboratory Animal Resources.

**In the 14-day** oral toxicity study, after a 8-day acclimation period, five Wistar rats of each sex (females were nulliparous and non-pregnant) received daily by oral gavage potassium polyaspartate (A-5D K/SD) at levels of 0, 60, 125, 250, 500 and 1000 mg/kg body weight for 14 days and were sacrificed on day 15 to evaluate signs of toxicity. A concurrent vehicle control group receiving analytical grade water at the dose of 10 mL/kg was also maintained. At the start of the experimental periods, animals utilized were 8 weeks old and weighed 151–190 g (males) and 130–157 g (females), Groups of five animals of similar sex were housed in each cage. The rats were examined daily for signs of toxicity, morbidity and mortality. Animals were subjected to detailed clinical examination before starting the study and weekly thereafter during the treatment period and at termination. Body weight and food consumption were recorded weekly. Laboratory investigations were performed on blood at termination of the study. All animals sacrificed terminally were subjected to a detailed necropsy and weights of kidneys, liver, adrenals, testes, spleen, brain and heart were recorded.

**In the 90-day** dietary study, after a period of 7 days for acclimation, groups of ten Wistar rats of each sex (females were nulliparous and non-pregnant) were administered potassium polyaspartate (A-5D K/SD) by oral gavage daily at levels of 250, 500 and 1000 mg/kg of body weight for 90 days. A concurrent control group of ten males and ten females receiving the vehicle, i.e. analytical grade water, at 5 mL/kg, was also maintained for 90 days. Additionally, groups of five rats per sex which had received the vehicle at 5 mL/kg and the test substance at the high dose level, i.e. 1000 mg/kg body weight, were further observed for a period of 28 days following the 90 days treatment, for assessment of reversibility, persistence or delayed occurrence of toxicity (Recovery). At the start of the experimental periods, animals utilized were 8 weeks old and weighed 151–196 g (males) and 130–160 g (females), Groups of two/three animals of similar sex were housed in each cage. All animals were observed for mortality twice daily. They were subjected to detailed clinical examinations once daily during the treatment and recovery periods. All signs of ill health, together with any behavioral changes or reaction to treatment were recorded for individual animals. General cage-side clinical examinations were made once daily during the treatment period and also during the recovery period. The rats were subjected to detailed clinical examinations before initiation of the treatment and weekly thereafter during the treatment and recovery periods Signs noted were included, but not limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity such as lacrimation, piloerection, pupil size, and unusual respiratory pattern. Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards) were also recorded. Ophthalmoscopic examination was conducted on control and high dose group animals before starting the study and at termination of treatment. A functional observation battery (FOB) was performed in the thirteenth week of treatment to assess parameters such as sensory reactions to various stimuli, grip strength and motor activity (Moser, 1989). Body weight and food consumption were recorded weekly.

After completion of 90 days of treatment (on day 91) and at termination of the reversal period (on day 119), blood samples were drawn, under light carbon dioxide anesthesia, from the orbital plexus. The samples were collected in tubes containing K-EDTA, for hematology, and heparin, for clinical chemistry, as anticoagulants. Animals were fasted overnight prior to blood collection, but had access to water ad libitum. Also, urine samples from all the rats were collected at termination of the treatment period and at the end of the reversal period. Blood samples were analyzed for hematologic parameters (hemoglobin (Hb), packed cell volume (PCV), total red cell count (total RBC), total white cell count (total WBC), platelets (PLT). The following calculated RBC associated indices were recorded: mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC). Differential leukocyte count (WBC) was conducted and leucocytes were differentiated as neutrophils (N), lymphocytes (L), monocytes (M), eosinophils (E) and basophils (B). After completion of 90 days of treatment (on day 91) and at termination of the reversal period (on day 119), coagulation parameters were determined: prothrombin time (PT) and activated partial thromboplastin time (APTT). After completion of 90 days of treatment (on day 91) and at termination of the recovery period (on day 119), plasma samples were analyzed for determination of clinical chemistry parameters: glucose (Glu), blood urea nitrogen (BUN), urea, total protein (Tot.Pro), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, albumin, globulin, total cholesterol; total bilirubin (TBIL), calcium, phosphorous, triglycerides (TG), triiodothyronine (T3), Thyroxine (T4), Thyroid Stimulating Hormone (TSH), sodium and potassium. Urinalysis was performed on all animals, few days before the treatment period (day 86) and just before the end of the

reversal period (day 116 and day 117). Urine samples were collected over a period of about 2–4 h during which no food and water were offered.

At termination of treatment (on day 90) and at termination of the reversal period (on day 118) the stage of oestrous cycle of all females was determined by taking vaginal smears.

All animals sacrificed terminally were subjected to a detailed necropsy and weights of kidneys, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries and heart were recorded. Histopathological evaluation was performed on all tissues [(brain, spinal cord, eye, pituitary, thyroid, parathyroid, spleen, thymus, adrenals, pancreas, trachea, lungs, heart, aorta, oesophagus, stomach, duodenum, Jejunum, terminal ileum, colon, rectum, liver, kidneys, urinary bladder, prostate, seminal vesicle, epididymides, testes, ovaries, uterus, skin. Sciatic nerve, bone marrow (smear), mammary gland (females), mesenteric lymph node, axillary lymph node and salivary glands)] in all rats from the control and high dose groups.

#### 2.3.3. Statistical analyses

The body weight, organ weight, hematology and clinical chemistry data of different groups were analyzed for statistical significance and evaluated for homogeneity of variances and normality by Bartlett's test (Bartlett, 1937). Where Bartlett's test indicated homogeneous intra-group variances, treated and control groups were compared using an one-way analysis of variance (ANOVA) (Snedecor and Cochran, 1980), followed by comparison of the treated groups to the control groups by Dunnett's pair wise comparison test (Scheffé, 1953), when 'F' value was significant. All analysis and comparisons were evaluated at $p < 0.05$.

## 3. Results

### 3.1. Genotoxicity studies

#### 3.1.1. Bacterial reverse mutation assay

The results of the bacterial reverse mutation test (Ames assay) indicate that the frequencies of histidine revertant colonies of *Salmonella typhimurium* at all concentrations of potassium polyaspartate (A-5D K/SD) in the strains TA1535, TA97a, TA98, TA100 and TA102, with and without the presence of a metabolic activation system (in experiment No.1 and No.2) were comparable to those observed in the vehicle control group, according to the criteria employed for evaluation of mutagenic potential (Table 3). Concurrent positive controls demonstrated sensitivity of the assay both in the presence and absence of metabolic activation. Plate counts for the spontaneous histidine revertant colonies in the vehicle control groups were found to be within the frequency ranges expected from the laboratory historical control data and all criteria for a valid study were met. Under the conditions of the study, potassium polyaspartate (A-5D K/SD) was not mutagenic in *Salmonella typhimurium* strains TA1535, TA97a, TA98, TA100 and TA102.

#### 3.1.2. In vitro mammalian cell micronucleus assay

In *in vitro* mammalian cell micronucleus assay, a comparison of the percentage incidence of micronucleated binucleated cells (BNCs) for each of the three experiments conducted with potassium polyaspartate (A-5D K/SD), either with or without the metabolic activation system, did not reveal any biologically significant or dose-related increase. Also, there was no incidence of a biologically significant increase in the percentage incidence of micronucleated BNCs at any of the concentration levels in the cultures treated with potassium polyaspartate (A-5D K/SD). Sensitivity of the test system and activity of S9 mix were demonstrated in the positive control group. The positive controls (i.e. directly acting clastogen mitomycin C, directly acting aneugen vinblastine and indirectly acting clastogen cyclophosphamide) induced significant increase in frequencies of micronucleated binucleated cells over the concurrent controls, which validated the test method. Assessment of the cytokinesis-block proliferation index (CPBI) made during the preliminary cytotoxicity test and the main study indicated that potassium polyaspartate (A-5D K/SD) exerted no cytotoxic effects on the cultured human lymphocytes in any of the three experiments conducted, both with and without metabolic activation system. Under the conditions of the assay, potassium polyaspartate (A-5D K/SD) did not induce any biologically significant and concentration related increase in the incidence of micronucleated (BNCs) over the tested range (Table 4). Therefore, these negative results indicate that potassium polyaspartate (A-5D K/SD) did not induce chromosome breaks and/or gain or loss (i.e. it is not clastogenic and aneugenic) in cultured mammalian cells (i.e. human peripheral blood lymphocytes).

### 3.2. Oral toxicity studies

#### 3.2.1. 14-Day repeated-dose oral toxicity study in rats

A 14-days range-finding study has been conducted in rats with A-5D K/SD to collect indication of target organs and help in selection of appropriate doses for a 90-day study. The study involved daily oral administration of A-5D K/SD to groups of Wistar rats, five per sex per dose, at the doses of 60, 125, 250, 500 and 1000 mg/kg body weight for 14 days to evaluate its toxicity.

No mortality was observed in rats treated with A-5D K/SD at and up to 1000 mg/kg body weight and in vehicle control group animals in a 14-days range-finding study.

In animals treated at and up to the dose of 1000 mg/kg, the daily general clinical examinations and weekly detailed clinical examinations did not reveal any treatment related incidence of clinical abnormalities.

The body weight gain by male and female rats treated at and up to 1000 mg/kg body weight was found to be comparable to that by the control rats throughout the treatment period. (Supplemental Figs. 1 and 2).

The values of average daily food consumption were not affected in both male and female rats treated at and up to 1000 mg/kg body weight. The average daily food consumption per rat per day, computed over the period of two weeks, by male rats receiving test substance at 60, 125, 250, 500 and 1000 mg/kg was 102%, 105%,107%, 106% and 105% respectively of that by control rats. Similarly, the average daily food consumption by female rats receiving the test substance at 60, 125, 250, 500 and 1000 mg/kg bw/day was 112%, 104%, 113%, 118% and 105% respectively of that by control rats (Supplemental Table 1).

At the end of treatment period, the group mean values of hematological parameters such as hemoglobin, packed cell volume (PCV), total RBC count, RBC indices and platelet count of male and female rats, treated with test substance at and up to the level of 1000 mg/kg, were found to be comparable with those of the control animals. Although a statistically significant change was seen in eosinophilic count for males of the 500 mg/kg bw/day group and for females of the 125 mg/kg bw/day group, it was not considered to be the treatment related change due to lack of dose related effect (Supplemental Table 2).

The test article, up to 1000 mg/kg bw/day dose, did not induce any changes in the plasma levels of total protein, albumin, globulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glucose, blood urea nitrogen (BUN), urea, creatinine and total cholesterol in male and female rats at termination of the treatment.

**Table 3**
Summary data on Histidine Revertant Colonies.

| Experiment No.1 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | Concentration | | TA1535 | | TA97a | | TA98 | | TA100 | | TA102 | |
| | (μg/plate) | S9 | Mean | ± SD | Mean | ± SD | Mean | ± SD | Mean | ± SD | Mean | ± SD |
| **A-5D K SD** | 5000 | – | 14.67 | 2.31 | 115.33 | 12.22 | 30.33 | 7.57 | 119.33 | 5.03 | 249.33 | 6.11 |
| | | + | 13.33 | 2.52 | 132.00 | 2.00 | 24.67 | 3.51 | 120.00 | 12.00 | 242.67 | 8.33 |
| | 1500 | – | 14.00 | 4.00 | 122.67 | 3.06 | 26.33 | 4.73 | 140.00 | 17.09 | 224.00 | 8.00 |
| | | + | 13.67 | 4.51 | 108.00 | 29.05 | 31.00 | 7.00 | 127.33 | 13.61 | 256.00 | 10.58 |
| | 500 | – | 21.00 | 3.61 | 125.33 | 5.03 | 21.00 | 4.00 | 130.00 | 19.08 | 237.33 | 4.62 |
| | | + | 17.67 | 3.51 | 111.33 | 22.03 | 29.67 | 6.11 | 121.33 | 11.02 | 248.00 | 4.00 |
| | 150 | – | 13.33 | 3.06 | 138.67 | 17.01 | 19.33 | 1.53 | 129.33 | 20.13 | 261.33 | 12.86 |
| | | + | 14.33 | 1.53 | 92.67 | 9.24 | 23.00 | 6.08 | 118.00 | 24.58 | 265.33 | 24.44 |
| | 50 | – | 15.33 | 4.51 | 94.67 | 18.58 | 23.00 | 2.00 | 134.67 | 7.02 | 250.67 | 6.11 |
| | | + | 17.00 | 2.65 | 124.00 | 2.00 | 19.33 | 3.21 | 158.67 | 18.58 | 261.33 | 10.07 |
| **Vehicle Control** | | | | | | | | | | | | |
| Analytical grade water | 100 μl | – | 14.00 | 2.65 | 138.67 | 17.01 | 28.33 | 6.43 | 127.33 | 20.13 | 266.67 | 32.58 |
| | | + | 21.00 | 7.00 | 115.33 | 8.33 | 23.33 | 4.93 | 123.33 | 14.19 | 252.00 | 8.00 |
| **Positive Controls** | | | | | | | | | | | | |
| Sodium azide | 2 | – | 840.00 | 32.74 | – | – | – | – | – | – | – | – |
| ICR 191 | 1 | – | – | – | 1008.00 | 141.53 | – | – | – | – | – | – |
| 4-Nitroquinoline-N-oxide | 0.5 | – | – | – | – | – | 490.67 | 18.90 | – | – | – | – |
| 3-Methylmethane Sulphonate | 1 μl | – | – | – | – | – | – | – | 1152.00 | 174.36 | 2232.00 | 119.47 |
| 2-Aminoanthracene | 10 | + | 257.33 | 30.29 | – | – | – | – | – | – | – | – |
| 2-Aminofluorene | 20 | + | – | – | 1210.67 | 76.87 | 617.33 | 58.01 | 1496.00 | 170.27 | – | – |
| Danthron | 30 | + | – | – | – | – | – | – | – | – | 2133.33 | 216.20 |
| **Experiment No.2** | | | | | | | | | | | | |
| **Treatment** | **Concentration** | | **TA1535** | | **TA97a** | | **TA98** | | **TA100** | | **TA102** | |
| | **(μg/plate)** | **S9** | **Mean** | **± SD** | **Mean** | **± SD** | **Mean** | **± SD** | **Mean** | **± SD** | **Mean** | **± SD** |
| **A-5D K SD** | 5000 | – | 11.67 | 5.03 | 116.00 | 9.17 | 39.33 | 2.52 | 138.00 | 6.00 | 256.00 | 6.93 |
| | | + | 16.33 | 1.15 | 116.00 | 6.00 | 40.33 | 9.07 | 133.33 | 9.45 | 273.33 | 14.05 |
| | 15000 | – | 11.33 | 2.52 | 113.33 | 8.33 | 32.67 | 2.52 | 140.00 | 5.29 | 256.00 | 8.00 |
| | | + | 12.00 | 3.46 | 122.00 | 5.29 | 34.67 | 1.53 | 136.67 | 8.08 | 258.67 | 6.11 |
| | 500 | – | 11.00 | 6.00 | 108.67 | 6.11 | 39.33 | 4.93 | 134.00 | 4.00 | 272.00 | 6.93 |
| | | + | 11.33 | 3.06 | 118.67 | 6.43 | 40.00 | 9.17 | 135.33 | 5.03 | 261.33 | 14.05 |
| | 150 | – | 12.67 | 3.79 | 107.33 | 1.15 | 33.67 | 2.31 | 140.67 | 4.16 | 253.33 | 6.11 |
| | | + | 13.00 | 1.73 | 120.67 | 3.06 | 36.33 | 6.03 | 134.67 | 3.06 | 280.00 | 8.00 |
| | 50 | – | 11.00 | 1.00 | 117.33 | 3.06 | 32.33 | 2.08 | 138.67 | 6.43 | 274.67 | 14.05 |
| | | + | 12.00 | 0.00 | 119.33 | 3.06 | 40.00 | 9.17 | 142.00 | 5.29 | 260.00 | 4.00 |
| **Vehicle Control** | | | | | | | | | | | | |
| Analytical grade water | 100 μl | – | 10.00 | 4.58 | 118.67 | 3.06 | 37.67 | 2.52 | 136.00 | 7.21 | 266.67 | 12.22 |
| | | + | 14.67 | 1.15 | 120.00 | 4.00 | 32.33 | 2.52 | 134.67 | 1.15 | 257.33 | 10.07 |
| **Positive Controls** | | | | | | | | | | | | |
| Sodium azide | 2 | – | 865.33 | 22.03 | – | – | – | – | – | – | – | – |
| ICR 191 | 1 | – | – | – | 1160.00 | 65.48 | – | – | – | – | – | – |
| 4-Nitroquinoline-N-oxide | 0.5 | – | – | – | – | – | 533.33 | 26.03 | – | – | – | – |
| 3-Methylmethane Sulphonate | 1 μl | – | – | – | – | – | – | – | 1266.67 | 88.12 | 2357.33 | 75.61 |
| 2-Aminoanthracene | 10 | + | 629.33 | 52.20 | – | – | – | – | – | – | – | – |
| 2-Aminofluorene | 20 | + | – | – | 1330.67 | 53.27 | 666.67 | 20.13 | 1618.67 | 222.04 | – | – |
| Danthron | 30 | + | – | – | – | – | – | – | – | – | 2197.33 | 153.12 |
| | | S9: | – | Without S9 | | + | With S9 | | | | | |

Although a statistically significant decrease in values of aspartate aminotransferase (AST) was observed for males of the 125 and 1000 mg/kg bw/day groups; a statistically significant increase in glucose for males of the 250 mg/kg bw/day group; a statistically significant increase in blood urea nitrogen (BUN) and urea was observed for males of the 500 mg/kg bw/day group; slight but statistically significant increase in albumin was observed for females of the 500 and 1000 mg/kg bw/day groups, a statistically significant decrease in ALP for females of the 1000 mg/kg bw/day group and a statistically significant increase in total cholesterol for females of the 125 and 1000 mg/kg bw/day groups. However, it was not considered to be the treatment related change due to lack of dose related effect. Few individual deviations were noted both in treatment and control groups and hence none were considered of toxicological significance (Supplemental Table 3).

The values of absolute and relative weights of kidneys, liver, adrenals, testes, spleen, brain and heart of male/female rats treated with the test substance at and up to 1000 mg/kg bw/day dose were found to be comparable to those of the control group rats at termination of the treatment period. Although a statistically significant change was seen in absolute weights (male-liver and brain and female-kidneys and brain) and relative organ weights (male-kidneys and liver and female-kidney and brain), it was not considered to be the treatment related change due to lack of dose related effect (Supplemental Tables 4 and 5).

No gross pathological alterations were encountered in the rats sacrificed at termination of the study.

Based on these findings, the doses selected for the 'Repeated Dose 90 Days Oral Toxicity Study of A-5D K/SD in Wistar Rat were 250, 500 and 1000 mg/kg bw per day.

**Table 4**
Summary of incidence of micronucleated BNCs and cytotoxicity.

| Group/ Concentration (μg/ml) | Dose μg/ml | No. of Cells Analyzed | No. of BNC with MN | % of BNC with MN | CBPI | Cytostasis % |
|---|---|---|---|---|---|---|
| **Exp 1_Cultured Lymphocytes Treated for 3 h without Metabolic Activation** | | | | | | |
| Negative Control 0.9% Saline (w/v) | – | 2012 | 2 | 0.10 | 1.95 | – |
| Vehicle Control Analytical grade water | – | 2033 | 4 | 0.20 | 1.94 | – |
| Positive Control MMC | 0.8 | 2249 | 31 | 1.38* | 1.51 | 45.43 |
| Positive Control VBL | 0.08 | 2009 | 37 | 1.84* | 1.56 | 40.55 |
| A-5D K SD | 5000 | 2016 | 6 | 0.30 | 1.88 | 6.17 |
| | 1500 | 2037 | 6 | 0.29 | 1.90 | 4.73 |
| | 500 | 2033 | 4 | 0.20 | 1.88 | 6.48 |
| **Exp 2_Cultured Lymphocytes Treated for 20 h without Metabolic Activation** | | | | | | |
| Negative Control 0.9% Saline (w/v) | – | 2003 | 7 | 0.35 | 1.97 | – |
| Vehicle Control Analytical grade water | – | 2000 | 7 | 0.35 | 1.90 | – |
| Positive Control MMC | 0.8 | 2017 | 37 | 1.83* | 1.56 | 37.45 |
| Positive Control VBL | 0.08 | 2000 | 34 | 1.70* | 1.62 | 31.14 |
| A-5D K SD | 5000 | 2170 | 6 | 0.28 | 1.89 | 1.10 |
| | 1500 | 2079 | 5 | 0.24 | 1.90 | - 0.33 |
| | 500 | 2000 | 6 | 0.30 | 1.87 | 2.53 |
| **Exp 3_Cultured Lymphocytes Treated for 3 h with Metabolic Activation** | | | | | | |
| Negative Control 0.9% Saline (w/v) | – | 2009 | 6 | 0.30 | 1.92 | – |
| Vehicle Control Analytical grade water | – | 2065 | 4 | 0.19 | 1.86 | – |
| Positive Control CPM | 6.25 | 2013 | 40 | 2.00* | 1.72 | 16.92 |
| A-5D K SD | 5000 | 2013 | 10 | 0.50 | 1.85 | 0.84 |
| | 1500 | 2019 | 5 | 0.25 | 1.84 | 1.85 |
| | 500 | 2008 | 5 | 0.25 | 1.85 | 0.99 |

*$p < 0.05$.

*3.2.2. 90-Day repeated-dose oral toxicity study in rats*

Afterwards, a subchronic toxicity study has been conducted in rats with A-5D K/SD for a period of 90 days (OECD 408), modified to include assessment of some additional parameters described in the more recent guideline on repeated-dose 28-day oral toxicity study in rodents (OECD 407). The additional parameters place more emphasis on endocrine-related endpoints, (e.g. determination of thyroid hormones, gross necropsy and histopathology of tissues that are indicators of endocrine-related effects) and assessment of oestrous cycles. The modified 90-day study should allow for the identification of chemicals with the potential to cause neurotoxic, immunological, reproductive organ effects or endocrine-mediated effects, which may warrant further in-depth investigation.

This study involved daily oral administration of A-5D K/SD to groups of Wistar rats 10 per sex per dose, at the doses of 250, 500 and 1000 mg/kg of body weight for 90 days to evaluate its toxicity and reversibility of toxicity, if any.

*3.2.2.1. Mortality, body weights, food consumption, ophthalmoscopy and neurobehavioral assessment.* There was no incidence of any treatment related mortality amongst the rats treated with the test substance at any of the tested dose levels. All treated animals survived throughout the treatment period of 90 days and also during the recovery period.

The daily general clinical examinations and weekly detailed clinical examinations did not reveal any remarkable and treatment related incidence of clinical abnormalities.

Body weight gain by male and female rats treated with the test substance at and up 1000 mg/kg bw/day was found to be comparable to that by the control group throughout the treatment period (Figs. 3 and 4). Also during recovery period, the weight gain by male and female rats from the high dose group was found to be comparable to that by the control group rats.

The values of average daily food consumption by male and female rats treated with the test substance at different dose levels, remained comparable to those of the control group rats. The average daily food consumption per rat per day, computed over the period of 13 weeks, by male rats of group 250, 500 and 1000 mg/kg bw/day groups was 99%, 98% and 97% of that by control rats. Similarly, the average daily food consumption by female rats of group 250, 500 and 1000 mg/kg bw/day was 98%, 99% and 103% respectively of that by control rats. After cessation of treatment the values of food intake during the recovery period were found to be comparable among the vehicle control and the high dose groups (Table 5).

The terminal ophthalmologic examinations did not reveal any remarkable and treatment related incidence of ocular abnormalities (data not shown).

The neurological examinations (functional observations) conducted in the thirteenth week of the study did not reveal any remarkable and treatment related incidence of neurological abnormalities. Also no findings, indicative of a neurotoxic potential of the test article, were encountered during these examinations (data not shown).

*3.2.2.2. Hematology, coagulation, clinical chemistry, urinalysis and oestrous cycle.* At the end of the treatment period and also at the end of the recovery period, the group mean values of hematological parameters such as hemoglobin, packed cell volume, total and differential leukocyte counts, total RBC count, RBC indices, platelet count, activated partial thromboplastin time and prothrombin time of male and female rats, treated with the test substance at and up to the level of 1000 mg/kg of body weight, were found to be comparable with those of the control animals. Significant decrease in the value of neutrophils was observed in high dose group males as compared to control animals. The decrease in the value of neutrophils is marginal and not dose dependent and hence was not considered to be biologically significant (Table 6).

The A-5D K/SD, up to the dose level of 1000 mg/kg bw per day, did not induce any changes in the plasma levels of total protein, albumin, globulin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose, blood urea nitrogen (BUN), urea, creatinine,

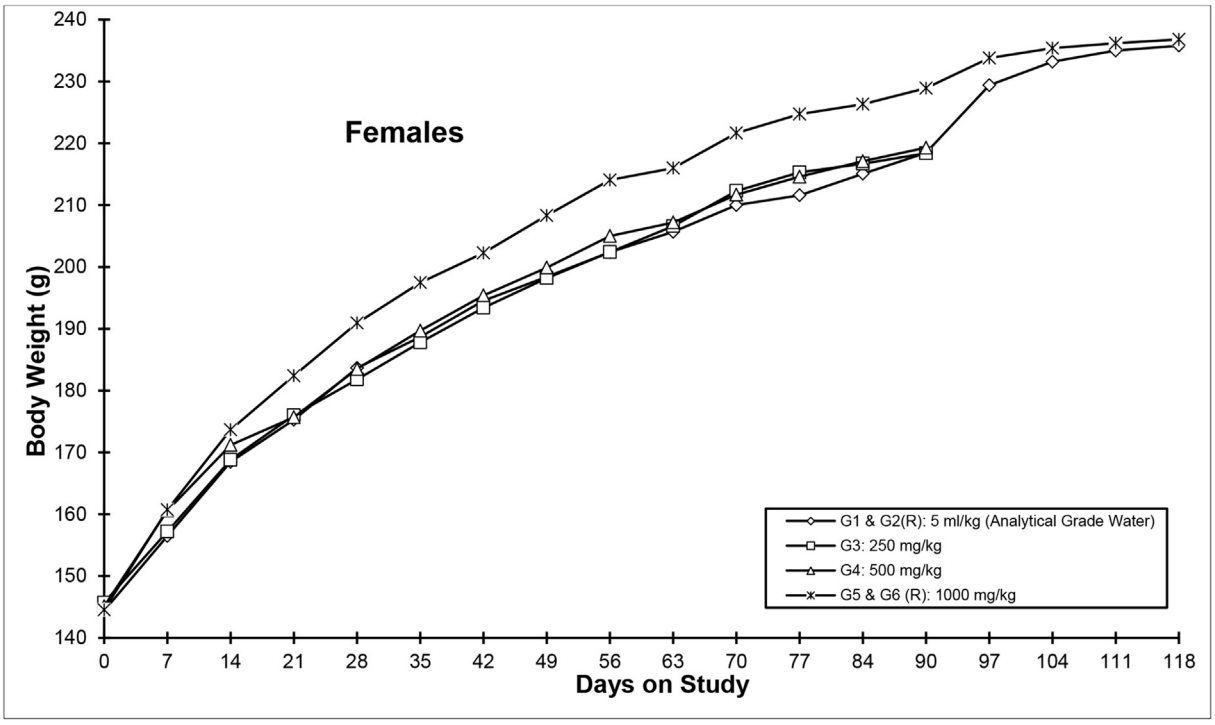


**Fig. 3.** Mean body weight - female rats administrated A-5D K/SD for 90 days.

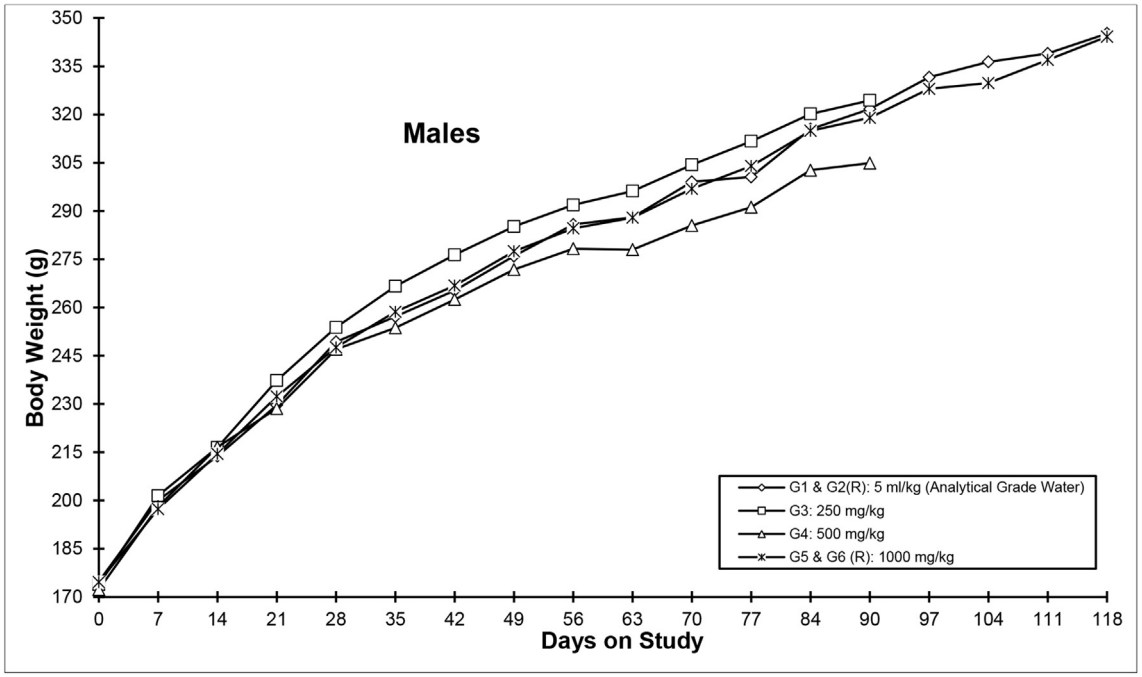


**Fig. 4.** Mean body weight - male rats administrated A-5D K/SD for 90 days.

total cholesterol, total bilirubin, sodium, potassium, calcium, phosphorous, thyroid hormones (T3, T4 and TSH) and triglycerides in male and female rats, at termination of the treatment and at the end of recovery period. The T4 values in three males and two females were below detectable range. This was observed in animals from treatment groups as well as from vehicle control group and not considered as treatment related change. Although a statistically significant change (significantly lower) was seen in total cholesterol values in high dosed males, it was not considered to be a treatment related change due to lack of dose related effect. The decrease in the cholesterol values was marginal and incidental and of no toxicological significance (Table 7).

The data on urinalysis evaluated at termination of treatment and also at the end of the recovery period did not indicate any abnormality due to treatment with the test substance. The data in treated animals and control animals was found to be comparable

**Table 5**
Summary of food consumption (g/rat/day) in 90-day repeated oral toxicity study.

| Group (N = 10) | (mg/kg bw/d) | Daily food consumption (g/rat/day) | Weeks on study | | | | | | | | | | | | | | | Treatment Period Average[a] | Recovery Period Average[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | | |
| Male (N = 5) | | | | | | | | | | | | | | | | | | | | | |
| G1 & G2(R) | Control 5 ml/kg (Analytical Grade Water) | Average | 19.39 | 17.26 | 16.46 | 19.13 | 15.20 | 16.80 | 17.16 | 17.21 | 17.52 | 16.21 | 19.36 | 18.48 | 14.78 | 14.90 | 17.27 | 20.85 | 22.26 | 17.31 | 18.82 |
| G3 | 250 | Average | 19.76 | 17.35 | 16.17 | 18.75 | 15.04 | 16.81 | 16.48 | 16.87 | 17.59 | 16.53 | 19.30 | 17.51 | 14.72 | – | – | – | – | 17.14 | – |
| | | % of Control | 102 | 100 | 98 | 98 | 99 | 100 | 96 | 98 | 100 | 102 | 100 | 95 | 100 | – | – | – | – | 99 | – |
| G4: | 500 | Average | 19.26 | 17.18 | 16.28 | 18.92 | 14.68 | 16.17 | 16.83 | 16.49 | 17.45 | 15.99 | 19.27 | 18.17 | 14.09 | – | – | – | – | 16.98 | – |
| | | % of Control | 99 | 100 | 99 | 99 | 97 | 96 | 98 | 96 | 100 | 99 | 100 | 98 | 95 | – | – | – | – | 98 | – |
| G5 & G6(R) | 1000 | Average | 18.25 | 16.99 | 15.91 | 18.80 | 14.77 | 16.13 | 16.59 | 16.38 | 17.40 | 15.63 | 19.34 | 17.79 | 14.07 | 15.20 | 17.02 | 20.65 | 21.95 | 16.77 | 18.71 |
| | | % of Control | 94 | 98 | 97 | 98 | 97 | 96 | 97 | 95 | 99 | 96 | 100 | 96 | 95 | 102 | 99 | 99 | 99 | 97 | 99 |
| Female (N = 5) | | | | | | | | | | | | | | | | | | | | | |
| G1 & G2(R) | Control 5 ml/kg (Analytical Grade Water) | Average | 13.79 | 14.28 | 13.57 | 12.88 | 12.45 | 14.82 | 12.97 | 15.85 | 13.55 | 12.57 | 15.65 | 17.72 | 12.95 | 12.55 | 19.86 | 16.71 | 17.05 | 14.08 | 16.54 |
| G3 | 250 | Average | 13.78 | 13.67 | 13.13 | 11.40 | 11.92 | 13.94 | 12.68 | 15.49 | 13.35 | 12.45 | 15.76 | 19.36 | 13.21 | – | – | – | – | 13.86 | – |
| | | % of Control | 100 | 96 | 97 | 89 | 96 | 94 | 98 | 98 | 99 | 99 | 101 | 109 | 102 | – | – | – | – | 98 | – |
| G4 | 500 | Average | 15.46 | 13.78 | 13.95 | 12.13 | 11.55 | 13.83 | 12.76 | 15.58 | 12.50 | 12.52 | 15.76 | 18.10 | 13.35 | – | – | – | – | 13.94 | – |
| | | % of Control | 112 | 96 | 103 | 94 | 93 | 93 | 98 | 98 | 92 | 100 | 101 | 102 | 103 | – | – | – | – | 99 | – |
| G5 & G6(R) | 1000 | Average | 14.50 | 14.20 | 16.07 | 13.27 | 12.16 | 15.26 | 13.41 | 15.88 | 13.51 | 12.99 | 16.19 | 19.14 | 12.74 | 13.60 | 18.54 | 16.77 | 16.99 | 14.56 | 16.47 |
| | | % of Control | 105 | 99 | 118 | 103 | 98 | 103 | 103 | 100 | 100 | 103 | 103 | 108 | 98 | 108 | 93 | 100 | 100 | 103 | 100 |

[a] Study average based on food intake measured on weeks 1–13.
[b] Study average based on food intake measured on weeks 14–17.

(Supplemental Table 6).

Treatment with the test substance did not result in any remarkable and treatment related incidence of oestrous cycle abnormalities in any of the female rats. Females from all control and treated groups exhibited the normal pattern of oestrus cycle (data not shown).

*3.2.2.3. Organ weights.* The values of absolute and relative weights of kidneys, liver, adrenals, testes, epididymides, uterus, thymus, spleen, brain, ovaries and heart of male/female rats treated with the test substance were found to be comparable to those of the control group rats at termination of the treatment. Although statistically significant changes were observed in a few organs (increased weights of testes, brain and thymus) in high dose recovery male rats at the end of recovery period, the changes observed were considered to be incidental and not treatment related as similar changes were not observed in these organs in high dose male rats at the end of treatment period (Tables 8 and 9).

*3.2.2.4. Gross pathology and histopathology.* The test substance, at and up to the dose level of 1000 mg/kg bw/day, did not induce any remarkable and treatment related gross pathological alterations in any of the tissues of treated rats, as evident at the detailed necropsy examination carried out at termination of the study and also at the end of recovery period. The only incidental gross pathological finding observed was adhesion of stomach with liver in a male rat treated at the dose of 250 mg/kg body weight. This being an isolated finding, considered as incidental and not related to treatment (data not shown).

Histopathological examinations of the tissues of all rats from the control group and those treated at the high dose level did not reveal any significant and treatment related histopathological alterations. Some incidental and spontaneous lesions observed in animals from the vehicle control and high dose group (1000 mg/kg) were – perivascular lymphocytic aggregation, peribronchial lymphoid tissue hyperplasia and foam cells in lungs; portal lymphocytic infiltration, chronic inflammatory foci and necrosis in liver; tubular dilatation and interstitial nephritis in kidneys; chronic trachitis in trachea; sub-mucosal lymphoid hyperplasia in stomach, ileum and colon; isolated incidence of lymphocytic infiltration in rectum and adhesions to serosa of stomach. Also an isolated incidence of perivascular cuffing with lymphoid cells was observed in brain. All the microscopic changes noticed in this study appeared to be incidental as their frequency is very low and not dose dependent. Also the lesions were distributed with equal frequency in control and high dose animals and hence considered as incidental (Table 10).

Based on the findings of this study, the No-Observed-Adverse-Effect-Level (NOAEL) of A-5D K/SD in Wistar rats, following oral administration for 90 days was found to be equal to or greater than 1000 mg/kg bw.

No toxicity was observed at the maximum dose level of 1000 mg/kg bw/day (no toxicological hazard identified) in the 90 days study performed with A-5D K/SD in rats and a NOAEL of 1000 mg/kg bw/day has been identified for A-5D K/SD.

## 4. Discussion

The results of *in vitro* genotoxicity tests indicate that A-5D K/SD does not possess mutagenic or genotoxic potential under the conditions of the studies. In 14 and 90-day subchronic dietary studies, administration of A-5D K/SD at concentrations of 0, 250, 500 and 1000 mg/kg of body weight in the diet for 90 days does not produce any significant toxicologic manifestations. The A-5D K/SD was well tolerated at these dietary levels as evidenced by the absence of major treatment-related changes in the general condition and

**Table 6**
Hematology and coagulation values for male and female rats (mean ± SD) administered potassium polyaspartate (A-5D K/SD) in the diet for 90 days.

| | | G1 | G3 | G4 | G5 | G2#(R) | G6#(R) |
|---|---|---|---|---|---|---|---|
| | | 5 ml/kg Vehicle Control (Analytical grade water) | 250 mg/kg bw/day | 500 mg/kg bw/day | 1000 mg/kg bw/day | 5 ml/kg Vehicle Control (Analytical grade water) | 1000 mg/kg bw/day |
| Males | | | | | | | |
| | | n = 10 | n = 10 | n = 10 | n = 10 | n = 5 | n = 5 |
| Hb (g/dL) | | 15.28 ± 1.20 | 15.12 ± 0.70 | 15.38 ± 0.53 | 14.71 ± 1.11 | 15.98 ± 0.52 | 15.64 ± 0.63 |
| PCV (%) | | 42.24 ± 3.20 | 42.22 ± 1.95 | 42.23 ± 1.11 | 40.69 ± 2.95 | 44.64 ± 1.42 | 43.80 ± 1.62 |
| Total RBC ( × $10^6$/cmm) | | 8.93 ± 0.68 | 8.78 ± 0.43 | 9.09 ± 0.36 | 8.61 ± 0.67 | 9.32 ± 0.54 | 9.32 ± 0.13 |
| MCH (pg) | | 17.12 ± 0.73 | 17.23 ± 0.50 | 16.93 ± 0.57 | 17.10 ± 0.69 | 17.16 ± 0.47 | 16.79 ± 0.68 |
| MCV (fL) | | 47.33 ± 1.46 | 48.10 ± 1.34 | 46.48 ± 1.32 | 47.33 ± 2.34 | 47.95 ± 1.34 | 47.01 ± 1.79 |
| MCHC (g/dL) | | 36.17 ± 0.53 | 35.81 ± 0.37 | 36.41 ± 0.41 | 36.15 ± 0.43 | 35.80 ± 0.16 | 35.71 ± 0.33 |
| Total WBC ( × $10^3$/cmm) | | 10.16 ± 2.36 | 10.12 ± 2.90 | 12.48 ± 3.79 | 13.52 ± 3.81 | 12.17 ± 2.30 | 10.87 ± 3.05 |
| Differential WBC (%) | N | 29.53 ± 10.26 | 26.32 ± 5.80 | 25.13 ± 5.27 | 20.36 ± 5.61 * | 23.60 ± 6.09 | 22.72 ± 4.75 |
| | L | 67.51 ± 11.54 | 70.48 ± 6.65 | 72.49 ± 6.32 | 77.37 ± 5.88 | 73.48 ± 6.46 | 74.46 ± 5.98 |
| | M | 1.196 ± 2.005 | 0.982 ± 0.645 | 0.514 ± 0.216 | 0.542 ± 0.402 | 0.76 ± 0.49 | 0.70 ± 0.45 |
| | E | 1.632 ± 0.447 | 2.207 ± 1.337 | 1.867 ± 1.279 | 1.707 ± 0.810 | 2.11 ± 0.46 | 2.13 ± 0.89 |
| | B | 0.028 ± 0.058 | 0.024 ± 0.063 | 0.004 ± 0.007 | 0.016 ± 0.039 | 0.02 ± 0.02 | 0.00 ± 0.00 |
| PLT ( × $10^3$/cmm) | | 807.30 ± 224.52 | 853.60 ± 92.06 | 811.00 ± 128.74 | 838.50 ± 82.51 | 773.60 ± 106.03 | 673.00 ± 183.93 |
| PT (sec) | | 17.11 ± 0.97 | 17.57 ± 1.32 | 18.00 ± 1.40 | 17.76 ± 1.58 | 16.78 ± 1.25 | 17.18 ± 1.17 |
| APTT (sec) | | 13.91 ± 1.46 | 14.52 ± 2.05 | 13.63 ± 1.57 | 13.70 ± 0.68 | 12.58 ± 1.24 | 13.36 ± 1.83 |
| Females | | | | | | | |
| | | n = 10 | n = 10 | n = 10 | n = 10 | n = 5 | n = 5 |
| Hb (g/dL) | | 14.56 ± 0.68 | 14.70 ± 0.55 | 14.94 ± 0.54 | 14.90 ± 0.42 | 14.92 ± 0.77 | 15.22 ± 0.43 |
| PCV (%) | | 40.30 ± 1.43 | 40.68 ± 1.47 | 41.47 ± 1.46 | 40.92 ± 1.19 | 42.14 ± 2.01 | 42.48 ± 1.26 |
| Total RBC ( × $10^6$/cmm) | | 8.12 ± 0.36 | 8.19 ± 0.42 | 8.36 ± 0.51 | 8.27 ± 0.25 | 8.28 ± 0.34 | 8.53 ± 0.44 |
| MCH (pg) | | 17.94 ± 0.68 | 17.96 ± 0.54 | 17.90 ± 0.79 | 18.03 ± 0.61 | 18.02 ± 0.41 | 17.85 ± 0.53 |
| MCV (fL) | | 49.66 ± 1.45 | 49.70 ± 1.37 | 49.67 ± 1.82 | 49.52 ± 1.97 | 50.90 ± 1.12 | 49.83 ± 1.41 |
| MCHC (g/dL) | | 36.12 ± 0.56 | 36.14 ± 0.54 | 36.03 ± 0.51 | 36.42 ± 0.51 | 35.40 ± 0.34 | 35.83 ± 0.18 |
| Total WBC ( × $10^3$/cmm) | | 11.49 ± 4.20 | 11.96 ± 6.06 | 10.35 ± 5.01 | 9.60 ± 4.21 | 8.60 ± 1.92 | 9.17 ± 2.93 |
| Differential WBC (%) | N | 23.71 ± 6.82 | 24.75 ± 11.50 | 29.43 ± 10.00 | 24.94 ± 9.95 | 24.12 ± 8.92 | 24.74 ± 6.61 |
| | L | 72.84 ± 6.64 | 72.50 ± 11.26 | 68.58 ± 10.35 | 72.88 ± 10.44 | 72.86 ± 8.71 | 72.20 ± 7.88 |
| | M | 1.434 ± 2.170 | 0.786 ± 0.464 | 0.525 ± 0.291 | 0.721 ± 0.548 | 1.42 ± 1.08 | 1.53 ± 1.85 |
| | E | 1.908 ± 1.036 | 1.914 ± 1.362 | 1.443 ± 0.773 | 1.418 ± 0.728 | 1.48 ± 1.03 | 1.40 ± 0.66 |
| | B | 0.145 ± 0.410 | 0.058 ± 0.067 | 0.021 ± 0.042 | 0.035 ± 0.076 | 0.14 ± 0.24 | 0.11 ± 0.24 |
| PLT ( × $10^3$/cmm) | | 757.50 ± 164.13 | 852.30 ± 90.54 | 793.20 ± 141.68 | 797.10 ± 155.26 | 797.60 ± 142.90 | 852.40 ± 111.19 |
| PT (sec) | | 16.59 ± 1.40 | 16.36 ± 0.78 | 16.86 ± 0.88 | 16.98 ± 1.84 | 16.34 ± 0.73 | 16.46 ± 0.80 |
| APTT (sec) | | 11.23 ± 1.52 | 10.49 ± 1.92 | 10.96 ± 1.13 | 10.46 ± 1.20 | 11.24 ± 1.45 | 11.66 ± 1.72 |

Abbreviations: Hb, hemoglobin; PCV, packed cell volume; total RBC, total red cell count; total white cell count (total WBC); MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cell; N, neutrophils; L, lymphocytes; M, monocytes; E, eosinophils; B, basophils; PLT, platelet; PT prothrombin time; APTT, activated partial thromboplastin time.
# - 119 day.
*$P < 0.05$.

**Table 7**
Serum clinical chemistry values for male and female rats (mean ± SD) administered potassium polyaspartate (A-5D K/SD) in the diet for 90 days.

| | G1 | G3 | G4 | G5 | G2#(R) | G6#(R) |
|---|---|---|---|---|---|---|
| | 5 ml/kg Vehicle Control (Analytical grade water) | 250 mg/kg bw/day | 500 mg/kg bw/day | 1000 mg/kg bw/day | 5 ml/kg Vehicle Control (Analytical grade water) | 1000 mg/kg bw/day |
| Males | | | | | | |
| | n = 10 | n = 10 | n = 10 | n = 10 | n = 5 | n = 5 |
| Total Protein (g/dl) | 7.02 ± 0.28 | 6.99 ± 0.32 | 7.17 ± 0.28 | 7.01 ± 0.38 | 7.74 ± 0.74 | 7.60 ± 0.23 |
| Albumin (g/dl) | 1.71 ± 0.13 | 1.66 ± 0.12 | 1.67 ± 0.13 | 1.68 ± 0.12 | 1.52 ± 0.16 | 1.58 ± 0.15 |
| Globulin (g/dl) | 5.31 ± 0.33 | 5.33 ± 0.36 | 5.50 ± 0.20 | 5.33 ± 0.33 | 6.22 ± 0.58 | 6.02 ± 0.36 |
| ALT (U/L) | 45.90 ± 6.14 | 41.10 ± 7.52 | 47.60 ± 7.40 | 46.80 ± 9.32 | 47.40 ± 2.51 | 49.40 ± 9.02 |
| AST (U/L) | 90.10 ± 7.11 | 90.90 ± 9.70 | 97.20 ± 11.66 | 94.70 ± 12.05 | 89.40 ± 8.35 | 96.00 ± 10.34 |
| ALP (U/L) | 176.70 ± 67.05 | 141.80 ± 62.76 | 180.10 ± 37.28 | 169.30 ± 88.44 | 171.00 ± 47.87 | 161.60 ± 73.35 |
| Glucose (mg/dL) | 101.50 ± 13.25 | 102.30 ± 12.46 | 96.70 ± 13.94 | 100.00 ± 13.23 | 127.60 ± 61.05 | 106.00 ± 7.62 |
| BUN (mg/dL) | 19.50 ± 2.27 | 19.10 ± 1.97 | 20.10 ± 3.11 | 20.30 ± 3.77 | 18.00 ± 3.16 | 18.60 ± 2.88 |
| Urea (mg/dL) | 41.73 ± 4.86 | 40.87 ± 4.21 | 43.01 ± 6.65 | 43.44 ± 8.07 | 38.52 ± 6.77 | 39.80 ± 6.17 |
| Creatinine (mg/dL) | 0.43 ± 0.07 | 0.44 ± 0.05 | 0.44 ± 0.07 | 0.42 ± 0.05 | 0.48 ± 0.11 | 0.41 ± 0.05 |
| CHOL (mg/dL) | 42.90 ± 5.74 | 43.10 ± 6.67 | 49.40 ± 8.37 | 35.40 ± 7.99* | 50.20 ± 13.27 | 40.80 ± 6.06 |
| TG (mg/dL) | 142.80 ± 51.35 | 122.90 ± 40.14 | 116.90 ± 37.17 | 105.80 ± 31.43 | 135.00 ± 72.89 | 128.20 ± 29.06 |
| TBIL (mg/dL) | 0.14 ± 0.07 | 0.17 ± 0.08 | 0.18 ± 0.06 | 0.18 ± 0.08 | 0.12 ± 0.04 | 0.16 ± 0.05 |
| Na (mmol/L) | 146.50 ± 0.90 | 146.36 ± 1.14 | 147.65 ± 1.17 | 145.96 ± 1.34 | 145.96 ± 0.46 | 145.06 ± 0.96 |
| K (mmol/L) | 4.85 ± 0.45 | 4.69 ± 0.35 | 4.58 ± 0.34 | 4.95 ± 0.35 | 4.53 ± 0.35 | 4.70 ± 0.18 |
| Ca (mmol/L) | 10.56 ± 0.22 | 10.68 ± 0.24 | 10.69 ± 0.17 | 10.74 ± 0.29 | 10.38 ± 0.22 | 10.46 ± 0.21 |
| PHS (mg/dL) | 7.26 ± 1.05 | 7.53 ± 0.53 | 7.51 ± 0.67 | 7.77 ± 1.02 | 6.22 ± 0.24 | 6.66 ± 0.41 |
| T3 (ng/mL) | 1.48 ± 0.14 | 1.44 ± 0.16 | 1.33 ± 0.15 | 1.34 ± 0.12 | 1.69 ± 1.07 | 1.91 ± 0.62 |
| T4 (ng/mL) | 49.14 ± 7.15 | 51.87 ± 6.40 | 59.44 ± 30.44[a] | 48.91 ± 11.95[a] | 21.33 ± 0.97[a] | 28.58 ± 7.27 |
| TSH (pg/mL) | 3526.00 ± 1096.80 | 2982.00 ± 1155.62 | 3590.90 ± 2202.65 | 2796.00 ± 936.47 | 1407.00 ± 944.69 | 2304.00 ± 1035.39 |

(*continued on next page*)

**Table 7** (*continued*)

| | G1 | G3 | G4 | G5 | G2#(R) | G6#(R) |
|---|---|---|---|---|---|---|
| | 5 ml/kg Vehicle Control (Analytical grade water) | 250 mg/kg bw/day | 500 mg/kg bw/day | 1000 mg/kg bw/day | 5 ml/kg Vehicle Control (Analytical grade water) | 1000 mg/kg bw/day |
| Females | | | | | | |
| Total Protein (g/dl) | 7.33 ± 0.46 | 7.23 ± 0.52 | 7.22 ± 0.48 | 7.55 ± 0.58 | 7.76 ± 0.43 | 7.76 ± 0.43 |
| Albumin (g/dl) | 1.78 ± 0.17 | 1.86 ± 0.11 | 1.90 ± 0.12 | 1.79 ± 0.27 | 1.84 ± 0.18 | 1.96 ± 0.24 |
| Globulin (g/dl) | 5.55 ± 0.45 | 5.37 ± 0.50 | 5.32 ± 0.51 | 5.76 ± 0.52 | 5.92 ± 0.27 | 5.54 ± 0.52 |
| ALT (U/L) | 38.20 ± 5.75 | 38.60 ± 6.85 | 39.60 ± 6.22 | 41.50 ± 12.79 | 38.00 ± 55.73[b] | 33.80 ± 5.54 |
| AST (U/L) | 96.20 ± 10.42 | 87.10 ± 12.31 | 87.20 ± 11.35 | 98.50 ± 17.95 | 92.00 ± 88.29[b] | 78.60 ± 12.58 |
| ALP (U/L) | 75.80 ± 17.76 | 66.80 ± 25.09 | 91.00 ± 23.24 | 96.40 ± 94.41 | 79.25 ± 15.00[b] | 95.40 ± 38.44 |
| Glucose (mg/dL) | 100.10 ± 8.49 | 99.30 ± 9.79 | 99.80 ± 9.64 | 98.20 ± 9.07 | 106.20 ± 4.76 | 108.20 ± 7.26 |
| BUN (mg/dL) | 21.10 ± 2.73 | 20.40 ± 3.31 | 18.80 ± 2.49 | 19.00 ± 2.16 | 18.80 ± 2.86 | 18.60 ± 1.52 |
| Urea (mg/dL) | 45.15 ± 5.83 | 43.66 ± 7.08 | 40.23 ± 5.32 | 40.66 ± 4.62 | 40.23 ± 6.13 | 39.80 ± 3.25 |
| Creatinine (mg/dL) | 0.45 ± 0.05 | 0.44 ± 0.05 | 0.41 ± 0.04 | 0.43 ± 0.07 | 0.42 ± 0.04 | 0.38 ± 0.06 |
| CHOL (mg/dL) | 40.40 ± 10.05 | 44.90 ± 12.87 | 36.70 ± 7.83 | 43.40 ± 8.09 | 58.40 ± 8.02 | 41.40 ± 6.58 |
| TG (mg/dL) | 87.10 ± 22.48 | 85.00 ± 16.91 | 95.40 ± 21.94 | 95.60 ± 27.30 | 91.80 ± 18.16 | 102.60 ± 16.38 |
| TBIL (mg/dL) | 0.20 ± 0.07 | 0.14 ± 0.05 | 0.16 ± 0.07 | 0.18 ± 0.06 | 0.20 ± 0.07 | 0.20 ± 0.10 |
| Na (mmol/L) | 145.16 ± 1.99 | 145.31 ± 1.57 | 145.10 ± 3.02 | 145.62 ± 1.69 | 144.46 ± 0.50 | 145.90 ± 1.17 |
| K (mmol/L) | 4.42 ± 0.24 | 4.58 ± 0.32 | 4.37 ± 0.35 | 4.64 ± 0.32 | 4.57 ± 0.44 | 4.42 ± 0.05 |
| Ca (mmol/L) | 10.69 ± 0.24 | 10.74 ± 0.34 | 10.87 ± 0.34 | 10.78 ± 0.36 | 10.56 ± 0.40 | 10.60 ± 0.27 |
| PHS (mg/dL) | 5.82 ± 0.78 | 6.22 ± 0.86 | 6.06 ± 1.10 | 5.91 ± 0.89 | 5.46 ± 1.04 | 5.92 ± 0.61 |
| T3 (ng/mL) | 1.24 ± 0.15[c] | 1.51 ± 0.54 | 1.39 ± 0.23 | 1.46 ± 0.24 | 1.72 ± 0.49 | 1.84 ± 0.49 |
| T4 (ng/mL) | 45.43 ± 31.20 | 57.77 ± 15.40 | 41.28 ± 12.48 | 43.50 ± 14.77 | 46.38 ± 38.37[a] | 50.63 ± 45.19[a] |
| TSH (pg/mL) | 1774.00 ± 1753.44 | 3416.00 ± 2285.25 | 2466.00 ± 1845.29 | 3192.00 ± 1469.94 | 1745.20 ± 1451.25 | 1321.80 ± 932.21 |

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; CHOL, total cholesterol; TG, triglycerides; TBIL, Total Bilirubin; T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.
# - 119 day.
*$P < 0.05$.
[a] Value of T4 for one animal is not in detectable range.
[b] n = 4.
[c] n = 9.

**Table 8**
Absolute organ weights (g) for male and female rats (mean ± SD) administered potassium polyaspartate (A-5D K/SD) in the diet for 90 days.

| | G1 | G3 | G4 | G5 | G2#(R) | G6#(R) |
|---|---|---|---|---|---|---|
| | 5 ml/kg Vehicle Control (Analytical grade water) | 250 mg/kg bw/day | 500 mg/kg bw/day | 1000 mg/kg bw/day | 5 ml/kg Vehicle Control (Analytical grade water) | 1000 mg/kg bw/day |
| Males | | | | | | |
| | n = 10 | n = 10 | n = 10 | n = 10 | n = 5 | n = 5 |
| Fasting body weight (g) | 301.50 ± 22.29 | 305.60 ± 35.90 | 284.60 ± 20.37 | 296.90 ± 19.24 | 326.60 ± 44.22 | 326.20 ± 26.46 |
| Adrenals | 0.049 ± 0.005 | 0.047 ± 0.008 | 0.049 ± 0.009 | 0.049 ± 0.012 | 0.046 ± 0.010 | 0.043 ± 0.004 |
| Testes | 3.34 ± 0.43 | 3.29 ± 0.25 | 3.23 ± 0.29 | 3.27 ± 0.34 | 3.22 ± 0.20 | 3.75 ± 0.28* |
| Kidneys | 2.36 ± 0.35 | 2.38 ± 0.31 | 2.51 ± 0.50 | 2.33 ± 0.23 | 2.48 ± 0.32 | 2.47 ± 0.28 |
| Liver | 8.59 ± 1.03 | 8.52 ± 1.12 | 8.65 ± 0.86 | 8.72 ± 0.95 | 9.80 ± 1.79 | 9.47 ± 1.07 |
| Brain | 2.02 ± 0.07 | 1.95 ± 0.10 | 1.94 ± 0.17 | 1.97 ± 0.08 | 1.86 ± 0.19 | 2.02 ± 0.09* |
| Thymus | 0.23 ± 0.08 | 0.25 ± 0.06 | 0.18 ± 0.03 | 0.23 ± 0.04 | 0.16 ± 0.05 | 0.21 ± 0.03* |
| Heart | 1.03 ± 0.08 | 1.07 ± 0.13 | 1.02 ± 0.11 | 1.03 ± 0.08 | 1.08 ± 0.17 | 1.09 ± 0.08 |
| Spleen | 0.60 ± 0.08 | 0.59 ± 0.10 | 0.60 ± 0.12 | 0.65 ± 0.20 | 0.68 ± 0.21 | 0.66 ± 0.11 |
| Epididymides | 1.12 ± 0.12 | 1.16 ± 0.12 | 1.17 ± 0.07 | 1.15 ± 0.11 | 1.09 ± 0.07 | 1.19 ± 0.11 |
| Females | | | | | | |
| | n = 10 | n = 10 | n = 10 | n = 10 | n = 5 | n = 5 |
| Fasting body weight (g) | 194.90 ± 23.41 | 202.80 ± 12.69 | 200.00 ± 11.98 | 212.10 ± 25.74 | 222.80 ± 16.84 | 224.40 ± 19.24 |
| Adrenals | 0.062 ± 0.010 | 0.059 ± 0.008 | 0.061 ± 0.006 | 0.066 ± 0.008 | 0.072 ± 0.013 | 0.062 ± 0.005 |
| Ovaries | 0.147 ± 0.039 | 0.140 ± 0.018 | 0.148 ± 0.021 | 0.158 ± 0.026 | 0.164 ± 0.024 | 0.151 ± 0.015 |
| Kidneys | 1.63 ± 0.28 | 1.57 ± 0.15 | 1.75 ± 0.27 | 1.78 ± 0.36 | 1.75 ± 0.48 | 1.80 ± 0.13 |
| Liver | 6.19 ± 1.06 | 6.23 ± 0.50 | 6.58 ± 0.74 | 6.89 ± 1.01 | 7.30 ± 1.12 | 7.34 ± 0.82 |
| Brain | 1.84 ± 0.09 | 1.87 ± 0.08 | 1.90 ± 0.10 | 1.88 ± 0.10 | 1.92 ± 0.19 | 1.87 ± 0.08 |
| Thymus | 0.21 ± 0.06 | 0.21 ± 0.05 | 0.23 ± 0.06 | 0.25 ± 0.04 | 0.20 ± 0.02 | 0.23 ± 0.07 |
| Heart | 0.71 ± 0.09 | 0.77 ± 0.05 | 0.76 ± 0.05 | 0.79 ± 0.12 | 0.83 ± 0.09 | 0.84 ± 0.08 |
| Spleen | 0.48 ± 0.10 | 0.45 ± 0.07 | 0.48 ± 0.16 | 0.59 ± 0.18 | 0.50 ± 0.14 | 0.54 ± 0.10 |
| Uterus | 0.44 ± 0.11 | 0.46 ± 0.15 | 0.49 ± 0.14 | 0.57 ± 0.20 | 0.66 ± 0.25 | 0.63 ± 0.10 |

# - 119 day.
*$P < 0.05$.

appearance of the rats, neurobehavioral endpoints, growth, feed and water intake, ophthalmoscopic examinations, routine hematology and clinical chemistry parameters, urinalysis, or necropsy findings.

The No-Observed-Adverse-Effect-Level (NOAEL) of A-5D K/SD in Wistar rats, following oral administration for 90 days was found to be equal to or greater than 1000 mg/kg body weight, the highest dose tested.

**Table 9**
Relative organ weights (%) for male and female rats (mean ± SD) administered potassium polyaspartate (A-5D K/SD) in the diet for 90 days.

| | G1 | G3 | G4 | G5 | G2#(R) | G6#(R) |
|---|---|---|---|---|---|---|
| | 5 ml/kg Vehicle Control (Analytical grade water) | 250 | 500 | 1000 | 5 ml/kg Vehicle Control (Analytical grade water) | 1000 |
| | | mg/kg bw/day | mg/kg bw/day | mg/kg bw/day | | mg/kg bw/day |
| Males | | | | | | |
| | n = 10 | n = 10 | n = 10 | n = 10 | n = 5 | n = 5 |
| Adrenals | 0.016 ± 0.002 | 0.016 ± 0.002 | 0.017 ± 0.003 | 0.016 ± 0.004 | 0.014 ± 0.003 | 0.013 ± 0.001 |
| Testes | 1.11 ± 0.14 | 1.08 ± 0.09 | 1.14 ± 0.07 | 1.10 ± 0.11 | 0.99 ± 0.09 | 1.15 ± 0.09* |
| Kidneys | 0.78 ± 0.07 | 0.78 ± 0.05 | 0.88 ± 0.15 | 0.79 ± 0.07 | 0.76 ± 0.05 | 0.76 ± 0.04 |
| Liver | 2.84 ± 0.18 | 2.79 ± 0.22 | 3.04 ± 0.28 | 2.94 ± 0.25 | 2.99 ± 0.18 | 2.90 ± 0.21 |
| Brain | 0.67 ± 0.06 | 0.64 ± 0.05 | 0.68 ± 0.04 | 0.67 ± 0.04 | 0.57 ± 0.06 | 0.62 ± 0.04 |
| Thymus | 0.07 ± 0.03 | 0.08 ± 0.02 | 0.06 ± 0.01 | 0.08 ± 0.01 | 0.05 ± 0.01 | 0.06 ± 0.01* |
| Heart | 0.34 ± 0.02 | 0.35 ± 0.02 | 0.36 ± 0.04 | 0.35 ± 0.03 | 0.33 ± 0.02 | 0.33 ± 0.01 |
| Spleen | 0.20 ± 0.02 | 0.19 ± 0.03 | 0.21 ± 0.04 | 0.22 ± 0.07 | 0.20 ± 0.04 | 0.20 ± 0.03 |
| Epididymides | 0.37 ± 0.03 | 0.38 ± 0.02 | 0.41 ± 0.02 | 0.39 ± 0.04 | 0.34 ± 0.05 | 0.37 ± 0.05 |
| Females | | | | | | |
| | n = 10 | n = 10 | n = 10 | n = 10 | n = 5 | n = 5 |
| Adrenals | 0.032 ± 0.003 | 0.029 ± 0.004 | 0.030 ± 0.003 | 0.031 ± 0.005 | 0.032 ± 0.006 | 0.028 ± 0.003 |
| Ovaries | 0.076 ± 0.018 | 0.069 ± 0.007 | 0.074 ± 0.009 | 0.075 ± 0.015 | 0.074 ± 0.012 | 0.067 ± 0.007 |
| Kidneys | 0.83 ± 0.09 | 0.78 ± 0.08 | 0.87 ± 0.11 | 0.84 ± 0.13 | 0.79 ± 0.21 | 0.80 ± 0.03 |
| Liver | 3.17 ± 0.31 | 3.07 ± 0.19 | 3.29 ± 0.33 | 3.25 ± 0.24 | 3.28 ± 0.46 | 3.27 ± 0.21 |
| Brain | 0.96 ± 0.10 | 0.92 ± 0.05 | 0.95 ± 0.05 | 0.90 ± 0.07 | 0.87 ± 0.09 | 0.83 ± 0.05 |
| Thymus | 0.11 ± 0.03 | 0.10 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.02 | 0.09 ± 0.01 | 0.10 ± 0.02 |
| Heart | 0.36 ± 0.03 | 0.38 ± 0.03 | 0.38 ± 0.02 | 0.37 ± 0.04 | 0.37 ± 0.03 | 0.38 ± 0.03 |
| Spleen | 0.25 ± 0.04 | 0.23 ± 0.04 | 0.24 ± 0.08 | 0.28 ± 0.07 | 0.23 ± 0.06 | 0.24 ± 0.04 |
| Uterus | 0.22 ± 0.04 | 0.22 ± 0.07 | 0.25 ± 0.06 | 0.27 ± 0.10 | 0.30 ± 0.11 | 0.28 ± 0.02 |

# - 119 day.
*$P < 0.05$.

**Table 10**
Summary of histopathology observations for male and female rats administered potassium polyaspartate (A-5D K/SD) in the diet for 90 days.

| Tissue | Finding | Males | | Females | |
|---|---|---|---|---|---|
| | | G1 | G5 | G1 | G5 |
| | | 5 ml/kg Vehicle Control (Analytical grade water) | 1000 mg/kg bw/day | 5 ml/kg Vehicle Control (Analytical grade water) | 1000 mg/kg bw/day |
| | | n = 10 | n = 10 | n = 10 | n = 10 |
| Lungs | Perivascular lymphocytic aggregation | 2 | 2 | 1 | 1 |
| | Peribronchial lymphoid tissue hyperplasia | 1 | 2 | 1 | – |
| | Foam cells | 4 | – | – | 2 |
| Liver | Portal lymphocytic infiltration | 3 | 4 | 3 | 3 |
| | Chronic inflammatory foci | 1 | 1 | 2 | – |
| | Necrosis | 1 | 2 | – | 1 |
| Kidneys | Dilatation, tubular | 5 | 7 | 3 | 2 |
| | Nephritis, interstitial | – | 1 | 2 | 1 |
| Trachea | Chronic trachitis | 1 | 2 | – | 2 |
| Stomach | Sub-mucosal lymphoid hyperplasia | 1 | 1 | – | – |
| | Adhesions, serosal | – | – | – | 1 |
| Brain | Perivascular cuffing with lymphoid cells | 1 | – | – | – |
| Colon | Sub-mucosal lymphoid hyperplasia | 1 | 2 | 1 | 1 |
| Rectum | Sub-mucosal lymphoid hyperplasia | – | 2 | 1 | – |
| | Lymphocytic infiltration | – | 1 | – | – |
| Ileum | Sub-mucosal lymphoid hyperplasia | – | – | 1 | – |

The aforementioned toxicity studies were conducted as part of an investigation to examine the safety of A-5D K/SD for its proposed use as a stabiliser in wine, as fulfilling the requirements for the evaluation of the new food additive according to Regulation (EC) No 1331/2008.

## Conflict of interest

Gianni Triulzi and Jose Santos are employees of Esseco S.r.l. and Enartis USA respectively, which are both companies involved in manufacture of chemicals used in the wine industry.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fct.2017.09.036

## Transparency document

Transparency document related to this article can be found

online at https://doi.org/10.1016/j.fct.2017.09.036

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