

TSCA HEALTH & SAFETY STUDY COVER SHEET

15767

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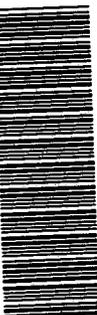
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<b>2.1 SUMMARY/ABSTRACT ATTACHED</b> (may be required for 8(e): optional for §4, 8(d) & FYI)  <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	<b>2.2 SUBMITTER TRACKING NUMBER OR INTERNAL ID</b> P917-006-754 99-2-1 File	<b>2.3 FOR EPA USE ONLY</b>						
<b>3.0 CHEMICAL/TEST SUBSTANCE IDENTITY</b> <input type="checkbox"/> Contains CBI <i>Reported Chemical Name (specify nomenclature if other than CAS name):</i> CAS# <u>148477-71-8</u> <u>3-(2,4-Dichlorophenyl)-2-oxo-1-oxaspiro (4.5)-3-en-4-yl ester 2,2-dimethyl-butanoic acid</u> Purity <u>99.1</u> % <input checked="" type="checkbox"/> Single Ingredient <input type="checkbox"/> Commercial/Tech Grade <input type="checkbox"/> Mixture Trade Name: <u>BAJ 2740</u> Common Name: _____ <table style="width:100%; border-collapse: collapse;"> <thead> <tr> <th style="width:30%; text-align: left;"><u>CAS Number</u></th> <th style="width:40%; text-align: left;"><u>NAME</u></th> <th style="width:30%; text-align: left;"><u>% WEIGHT</u></th> </tr> </thead> <tbody> <tr> <td colspan="3">Other chemical(s) present in tested mixture</td> </tr> </tbody> </table> <input type="checkbox"/> continuation sheet attached			<u>CAS Number</u>	<u>NAME</u>	<u>% WEIGHT</u>	Other chemical(s) present in tested mixture		
<u>CAS Number</u>	<u>NAME</u>	<u>% WEIGHT</u>						
Other chemical(s) present in tested mixture								
<b>4.0 REPORT/STUDY TITLE</b> <input type="checkbox"/> Contains CBI Subchronic Oral Toxicity Study in Mice and First Amendment to the Report. Report # 26536 Study # T7060885		 <b>BEHQ-99-14357</b>						
<b>5.1 STUDY/TSCATS INDEXING TERMS</b> [CHECK ONE] HEALTH EFFECTS (HE): <input checked="" type="checkbox"/> ENVIRONMENTAL EFFECTS (EE): _____ ENVIRONMENTAL FATE (EF): _____								
<b>5.2 STUDY/TSCATS INDEXING TERMS</b> (see instructions for 4 digit codes) STUDY TYPE: <u>STOX</u> SUBJECT ORGANISM (HE, EE only): <u>MICE</u> ROUTE OF EXPOSURE (HE only): <u>Oral</u> VEHICLE OF EXPOSURE (HE only): <u>FOOD</u> Other: _____ Other: _____ Other: _____								
<b>6.0 REPORT/STUDY INFORMATION</b> <input type="checkbox"/> Contains CBI <input type="checkbox"/> Study is GLP Laboratory <u>Bayer AG Toxicology Lab, Wuppertal, Germany</u> Report/Study Date <u>8/97</u> Source of Data/Study Sponsor (if different than submitter) _____ Number of pages _____ <input type="checkbox"/> continuation sheet attached								
<b>7.0 SUBMITTER INFORMATION</b> <input type="checkbox"/> Contains CBI Submitter: <u>Donald W. Lamb</u> Title: <u>VP, Product Safety &amp; Regulatory Affairs</u> Phone: <u>412-777-7431</u> Company Name: <u>Bayer Corporation</u> Company Address: <u>100 Bayer Road, Pittsburgh, PA. 15205</u> _____ Submitter Address (if different): _____ Technical Contact: <u>Same as above</u> Phone: ( ) _____ <input type="checkbox"/> continuation sheet attached								
<b>8.0 ADDITIONAL/OPTIONAL STUDY COMMENTS</b> <input type="checkbox"/> Contains CBI This compound is an experimental insecticide Note: There are random pages of the report. This is how it was received. We will send a follow-up completed report when we receive. <input type="checkbox"/> continuation sheet attached								

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### Continuation of 2.1

In a subchronic mouse study conducted with BAJ 2740, a distinct hypertrophy/activation of Leydig cells occurred at an increased incidence in male CD-1 mice dosed with 1000 ppm and 10,000 ppm of BAJ 2740. In the high-dose group, this finding showed increased severity and was accompanied by a vacuolation of Leydig cells in some animals.

### Abstract

A subchronic feeding toxicity with BAJ 2740 was performed in mice with concentrations of 0, 100, 1000, and 10,000 ppm in the diet in order to generate a data base for dose-selection for a subsequent oncogenicity study.

At 1000 ppm and above, an increased incidence of hypertrophic Leydig cells was found in the testes (1/1/9/10). This finding was characterized by a foamy cytoplasm of the enlarged Leydig cells and was located in the intertubular interstitium and beneath the capsule. In high-dose males (10,000 ppm) hypertrophy was increased in severity and accompanied by a minimal to slight vacuolation of the Leydig cells in some animals (0/0/0/7). Minimal degeneration of the germinal epithelium occurred in two animals in the low-dose group.

In the epididymides, minimal spermatic debris was seen in one animal in the intermediate-dose group. Prostates and seminal vesicles of all males were without any changes.

The NOAEL for male sexual organs was 100 ppm.

## STATUS OF PROJECT: BAJ 2740

### Project Management Toxicology

Results of a subchronic study in mice (T 7060885)  
(13 weeks)

Dose (ppm)	BW/FC	Clin. signs	Clinical chemistry/hematology	Organ weights	Histology
100	-	-	-	-	Liver: centrilobular hypertrophy (m)
1000	-	-	-	-	Liver: centrilobular hypertrophy (m) Adrenal cortex: cytoplasmic vacuolation (f) Testes*: Leydig cell hypertrophy, slight vacuolation (grade 1)
10000	-	-	Chol ↓ (f), Hb (↓) (f)	Kidneys: ↓ (m)	Liver: centrilobular hypertrophy (m/f), periportal cytoplasmic vacuolation (m/f) Adrenal cortex: cytoplasmic vacuolation (m/f) Testes*: Leydig cell hypertrophy, slight vacuolation (grade 2,3)

\* reexamination

**BAYER AG**  
TOXICOLOGY  
Friedrich-Ebert-Straße 217 - 333  
D-42096 Wuppertal

Report No.: 26536 A  
Report Date:

**BAJ 2740**

Subchronic Toxicity Study in CD-1 Mice  
(13-Week Feeding Study)

First Amendment to Report No. 26536 of August 13, 1997

by

Dr. K.H. Leser

Dr. A. Romeike

Study No. T 7060885

Page 1 of 12

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## **GLP Compliance Statement**

This amendment was conducted in compliance with the OECD Principles of Good Laboratory Practice (GLP, Bundesanzeiger No. 42a of March 2, 1983 and with the Principles of Good Laboratory Practice according to Annex 1 ChemG (Bundesgesetzblatt Part 1, July 29, 1994).

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Dr. K.H. Leser

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Date

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## Quality Assurance Statement

This amendment was audited by Quality Assurance on the dates given below. Audit reports have been submitted in writing to the study director and, if necessary, also the laboratory management, or other persons affected.

Date of audit:

Date of report to study  
director/management:

To the best of my knowledge, the results of the amendment have been correctly reported.

Quality Assurance Unit  
PH-QA-C/GLP, Bayer AG

Date: \_\_\_\_\_

Responsible: \_\_\_\_\_  
Dr. H. Lehn

## Signatures

Study Director:

\_\_\_\_\_  
(Dr. K.H. Leser)

\_\_\_\_\_  
Date

Study Pathologist:

\_\_\_\_\_  
(Dr. A. Romeike)

\_\_\_\_\_  
Date

Head of Cancerogenicity  
and Genotoxicity:

\_\_\_\_\_  
(Dr. E.M. Bomhard)

\_\_\_\_\_  
Date

### Reason for the Amendment

In a subacute toxicity study in dogs (feeding) with BAJ 2740 (dose levels: 0/400/2000/10000 ppm), the testes from males dosed at 2000 ppm and above revealed a bilaterally minimal vacuolation of the Leydig cells (0/0/2/2). In one high-dose animal this finding was combined with a hypertrophy/activation of the Leydig cells. Furthermore, the germinal epithelium of both testes and the prostate of this animal were considered to be slightly immature. The epididymides showed a massive oligo-/aspermia and slight spermatic debris.

After 13 weeks of treatment (subchronic toxicity study in dogs; feeding) with the test substance (dose levels: 0/200/630/2000 ppm), the testes from males of the intermediate (630 ppm) and high dose group (2000 ppm) revealed a slight to moderate, bilateral vacuolation of the Leydig cells (0/0/2/4). A hypertrophy/ activation of Leydig cells occurred in males after 630 ppm and above (0/0/2/3). Beside distinct vacuolation, also a foamy cytoplasm of enlarged Leydig cells was found. In addition, two high dose males had diffuse degeneration of the germinal epithelium (0/0/0/2). Aspermia and/or oligospermia were observed in the epididymides of males treated with 630 or 2000 ppm BAJ 2740 (0/0/3/2). Furthermore the prostates of one male of the intermediate dose group and of all males of the high-dose group were considered to be immature (0/0/1/4).

In both studies, the Leydig cell alterations were considered to result most probably from an impaired testosterone synthesis or metabolism. Degeneration of the germinal epithelium, oligo-/aspermia of the epididymides, and immaturity of the prostates were regarded as secondary to the Leydig cell vacuolation and its possible testosterone imbalances.

Upon the basis of the aforementioned findings in canine male sexual organs, male sexual organs (testes, epididymides, prostates, seminal vesicles) of CD-1 mice from the subchronic study under discussion (Pathology No. 4733) (dose levels: 0/100/1000/10000 ppm) were reexamined.

### Materials and Methods

Sections approximately 5 µm thick were prepared from testes, epididymides, prostate and seminal vesicles (all dose-groups) and stained with hematoxylin and eosin (H&E). The testes and epididymides slides of control and high dose animals were recut, to avoid staining differences between the slides of testes and epididymides (control and high-dose group) from the original study and the newly prepared ones.

### Results

The findings made during reexamination (additionally to the findings reported in the study report) are listed in Table 1 and 2.

At 1000 ppm and above an increased incidence of hypertrophic Leydig cells was found in the testes (1/1/9/10). This finding was characterized by a foamy cytoplasm of the enlarged Leydig cells and was located in the intertubular interstitium and beneath the capsule. In high-dose males (10000 ppm) hypertrophy/activation was increased in severity and accompanied by a minimal to slight vacuolation of the Leydig cells in some animals (0/0/0/7). Minimal degeneration of the germinal epithelium occurred in two animals of the low dose group.

In the epididymides, additionally to the findings in the study report, minimal spermatic debris was seen in one animal of the intermediate group. Prostates and seminal vesicles of all males were without findings.

### Conclusion

A distinct hypertrophy/activation of Leydig cells occurred at an increased incidence in male CD-1 mice dosed at 1000 ppm and 10000 ppm of BAJ 2740. In the high dose group, this finding showed an increased severity and was accompanied by a vacuolation of Leydig cells in some animals.

These Leydig cell alterations might indicate an altered testosterone synthesis or metabolism.

The no-(adverse) effect-level for male sexual organs was established at 100 ppm. Since a no-effect-level was not established in males due to hepatocellular hypertrophy these findings have no impact on the conclusions presented in the study report.

**Table 1: Additional findings in male sexual organs (Testes/Epididymides)**

Group	Dose ppm	Animal No.	Findings			
			Testes Hypertr./Activ. Leydig Cell	Testes Vacuolation Leydig Cell	Testes Degeneration Germ.Epith.	Epididymides Spermatic debris
1	0	1	-	-	-	-
		2	-	-	-	-
		3	-	-	-	-
		4	grade 1	-	-	-
		5	-	-	-	-
		6	-	-	-	-
		7	-	-	-	-
		8	-	-	-	-
		9	-	-	-	-
		10	-	-	-	-
2	100	11	-	-	-	-
		12	-	-	-	-
		13	grade 1	-	-	-
		14	-	-	-	-
		15	-	-	-	-
		16	-	-	grade (1	-
		17	-	-	grade 1	-
		18	-	-	-	-
		19	-	-	-	-
		20	-	-	-	-
3	1000	21	grade 1	-	-	-
		22	-	-	-	-
		23	grade 1	-	-	-
		24	grade 1	-	-	-
		25	grade 1	-	-	-
		26	grade 1	-	-	-
		27	grade 1	-	-	-
		28	grade 1	-	-	-
		29	grade 1	-	-	grade 1
		30	grade 1	-	-	-
		31	grade 2	grade 1	-	-
		32	grade 2	-	-	-
		33	grade 3	grade 1	-	-
		34	grade 3	grade 2	-	-

4	10000	35	grade 2	grade 1	-	-
		36	grade 3	grade 1	-	-
		37	grade 2	-	-	-
		38	grade 2	-	-	-
		39	grade 2	grade 1	-	-
		40	grade 2	grade 1	-	-

Hypertr. = Hypertrophy; Activ. = Activation; Germ.Epith. = Germinal Epithelium  
 grade 1 = minimal; grade 2 = slight; grade 3 = moderate; ( = unilaterally; - = no additional finding

**Table 2: Additional findings in male sexual organs (Prostates/Seminal Vesicles)**

Group	Dose ppm	Animal No.	Findings	
			Seminal vesicles	Prostate
1	0	1	-	-
		2	-	-
		3	-	-
		4	-	-
		5	-	-
		6	-	-
		7	-	-
		8	-	-
		9	-	-
		10	-	-
2	100	11	-	-
		12	-	-
		13	-	-
		14	-	-
		15	-	-
		16	-	-
		17	-	-
		18	-	-
		19	-	-
		20	-	-
3	1000	21	-	-
		22	-	-
		23	-	-
		24	-	-
		25	-	-
		26	-	-
		27	-	-
		28	-	-
		29	-	-
		30	-	-
		31	-	-
		32	-	-
		33	-	-
		34	-	-
		35	-	-

4	10000	36	-	-
		37	-	-
		38	-	-
		39	-	-
		40	-	-

grade 1 = minimal; grade 2 = slight; grade 3 = moderate; - = no additional finding

**Authentication**

The undersigned hereby declare that the histopathology data in this report were compiled by them, and that they reflect accurately the primary data records.

\_\_\_\_\_  
 Dr. A. Romeike  
 Study Pathologist

\_\_\_\_\_  
 Dr. V. Geiss  
 Cross-Check Pathologist

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**Pharmaceutical Business Group  
Elberfeld**

**Report No. Toxicology Report**

**Author(s):** Leser, K.H., Romeike, A

**BAJ 2740** Study on Subchronic Toxicity in CD-1-Mice  
(Administration in the Food over 13 Weeks)  
(Study-No. T7060885)

**Distribution: Full Report**  
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**Pharmaceutical Business Group  
Elberfeld**

**Report No.**

**Toxicology Report**

**Author(s):** Leser, K.H.  
Romeike, A

PH-PD Toxicology  
PH-PD Toxicology

**Title (English):**

**BAJ 2740** Study on Subchronic Toxicity in CD-1-Mice  
(Administration in the Food over 13 Weeks)  
(Study-No. T7060885)

**Title (Original Language):**

**International Study Register No. (ISRN):**

**Original Language:** Engl

**Version No.:** 1

**Translation:**

**Translation Version:**

**Study Completion Date:**

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**GLP COMPLIANCE STATEMENT**

This study was conducted in compliance with the OECD Principles of Good Laboratory Practice (GLP; German version published in: Bundesanzeiger No. 42a, March 2, 1983) and with the Principles of Good Laboratory Practice (GLP) according to Annex 1 ChemG (Bundesgesetzblatt Part I, July 29, 1994).

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Dr. Leser  
(Study Director)

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Date

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PATHOLOGY REPORTS (WITH AN ADDITIONAL SEPARATE PAGE NUMBERING)

**QUALITY ASSURANCE STATEMENT****Test Substance: BAJ 2740****Study No.: T7060885**

The study was audited by Quality Assurance on the dates stated below. Audit reports have been submitted in writing to the Study Director, to the laboratory management and, if necessary, to other persons affected.

Dates of audit	Dates of report to study director / management
05/07/1996 (study plan)	05/07/1996
05/14/1996	05/14/1996
07/29/1996	07/29/1996
08/05/1996	08/05/1996
08/07/1996	08/07/1996

To the best of my knowledge, the study results and methods used have been correctly reported.

Quality Assurance PH-QA-C/GLP, BAYER AG

Date: \_\_\_\_\_

Responsible: \_\_\_\_\_

(Dr. E. Niemers)

**SIGNATURES**

Study Director:

\_\_\_\_\_  
(Dr. Leser)

\_\_\_\_\_  
Date

Head of Cancerogenicity  
and Genotoxicity:

\_\_\_\_\_  
(Dr. Bomhard)

\_\_\_\_\_  
Date

Pathologist:

\_\_\_\_\_  
(Dr. Romeike)

\_\_\_\_\_  
Date

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## 1 SUMMARY

**BAJ 2740** was administered orally to CD-1 mice (10 males and 10 females per dose) via the food, in doses of 0 - 100 - 1000 - 10000 ppm over a period of about 13 weeks. This resulted in a test compound intake of 15.3, 163.8 and 1629.9 mg/kg body weight/day in males and 30.1, 233.6 and 2685.2 mg/kg body weight/day in females.

Mortality was not affected by treatment with **BAJ 2740**. Daily observations revealed no abnormalities with regard to body surfaces and orifices, general behavior, posture, respiration and excretory products in any of the animals up to and including doses of 10000 ppm.

In all treatment groups food consumption and water intake were comparable to that of controls.

Determination of body weight development did not reveal relevant differences to that of the control animals in mice up to and including 10000 ppm.

Hematological investigations revealed no relevant effects on the red and white blood cell counts or of any damage to the hematopoietic organs/tissues.

Clinical laboratory tests revealed slightly lower cholesterol concentrations in mice at 10000 ppm. Histopathological examination revealed hepatocellular hypertrophy in males at 100 ppm and above and in females at 10000 ppm. Periportal cytoplasmic vacuolation was found in males and females at 10000 ppm.

Cytoplasmic vacuolation was seen in the adrenal cortical cells in males at 10000 ppm and in females at 1000 ppm and above. Both, lower cholesterol concentrations as well as cytoplasmic vacuolation seen in liver and adrenal tissues are most likely related to the putative mechanism of action of **BAJ 2740**.

Clinical laboratory and histopathological examinations did not indicate nephrotoxic effects.

Gross and histopathological investigations of other organs and tissues gave no indication of test-compound-related functional or morphological changes.

Under the conditions described the administration of **BAJ 2740** was tolerated in female mice without treatment-related lesions at 100 ppm, for males there was no no-effect level due to hepatocellular hypertrophy. On the basis of the results obtained from this study the following dose levels were proposed for the subsequent oncogenicity study in mice: 0- ~~56~~- 280- 1400- 7000 ppm.

## 2 INTRODUCTION

**BAJ 2740** belongs to the group of cyclic ketoenols and is presently under development as an insecticide. The putative mechanism of action is an inhibition of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) reductase and an analogous enzyme in juvenile hormone synthesis during the larval stages of mites and insects.

This report describes the results of a subchronic toxicity study in which **BAJ 2740** was administered to mice orally via the diet for about 13 weeks.

The objective of the study was to provide information after prolonged and repeated exposure of **BAJ 2740** in mice which can be used in selecting dose levels for an oncogenicity in mice ("Dose-Range-Finding-Study").

These investigations were conducted in accordance with the guidelines listed below but clinical laboratory and histopathological determinations were reduced in accordance with the objective of the study.

"OECD guidelines for testing of chemicals, Section 4, health effects, No. 408, adopted 12th May 1981";

Council Recommendation (83/571/EEC), Annex I - Repeated Dose Toxicity; Official Journal of the European Communities L332, Nov. 11, 1983.

### 3 GENERAL INFORMATION

Table 1 - Key Study Dates

Study Identification:	
Test Number:	<b>T7060885</b>
Pathology Number:	<b>4733</b>
Delivery of Animals:	<b>April 30th, 1996</b>
Animal Age at Delivery:	<b>4 - 5 weeks</b>
Start of Study (First Day of Treatment):	<b>May 7th, 1996</b>
Animal Age at Study Start:	<b>5 - 6 weeks</b>
Mean Initial Weights at Study Start:	
Males:	<b>26.9 (23.9 - 28.8) g</b>
Females:	<b>23.8 (21.7 - 26.4) g</b>
Duration of Treatment:	<b>about 13 weeks</b>
Necropsy, End of Study (Last Animal Necropsied, End of Treatment):	<b>August 8th, 1996</b>

#### 3.1 Testing Facilities

The animal experiment, clinical laboratory examinations, necropsies, gross and histopathological investigations were conducted at the Institute of Toxicology, BAYER AG, Friedrich-Ebert-Straße 217-333, D-42096 Wuppertal, Germany.

#### 3.2 Duration of Study, Definition of Dates

The total duration of the animal experiment and other important dates are summarized in Table 1.

Unless indicated otherwise, the chronological dates in the report refer to months, weeks or days relative to the first day of treatment of the specified animal group. The dates given in data lists are defined as follows:

a) Body weights, clinical findings, food and water intake

Chronological information is given in weeks relative to the day of first administration (day 0). The data listed under day or week 0 were determined before the start of administration. Week 1, for example refers to the data determined on day 8 with a tolerance range of  $\pm 3$  days. For food intake and water intake the date in the lists corresponds to the week of weight determination. For calculating food or water consumption, the actual number of days with intake was taken into account.

If the test compound was administered in diet mixtures or drinking fluids the data on the test compound intake were calculated on the corresponding data of food or water consumption.

b) Lists of Surviving Animals

The number of animals still alive on the last day of the specified week is indicated.

c) Clinical Laboratory Investigations

The week is given in which the respective investigations were performed.

d) Organ Weights

In the summary tables of Report Part 1 as well as in Report Part 2 the necropsy dates are specified by the writing as "terminal sacrifice".

### 3.3 Archiving

The study protocol, original of the report, study documentation and raw data are held on file by BAYER AG. Materials (e.g. test compound, fixed tissue, histological sections) have also been retained.

### 3.4 Persons Involved, Responsibilities

Study Director:	Dr. Leser
Head of Cancerogenicity and Genotoxicity:	Dr. Bomhard
Test Compound Analyses:	Dr. Gau
Analysis of Test Compound in Carrier:	Dr. Rüngeler
Clinical Laboratory Determinations:	Dr. Loof
Head of Toxicologic Pathology:	Prof. Dr. Karbe
Gross Pathology:	Dr. Rinke
Histopathology:	Dr. Romeike
Laboratory Animal Services and Laboratory Equipment Services:	Dr. Hoffmann
Husbandry:	Dr. Leser
Technical Engineering:	Mr. Lömker, grad. engineer
Computer-assisted Data Processing:	Dr. Klotz
Archiving:	Prof. Dr. Schlüter
Quality Assurance:	Dr. Lehn

## 4 MATERIALS, TEST SYSTEM, METHODS

Table 2 - Test Substance, Vehicle and Administration Data

Test Substance:	<b>BAJ 2740</b>
Producer:	BAYER AG
Common Name:	none
Chemical name:	3-(2,4-Dichlorophenyl)-2-oxo-1-oxaspiro<4.5>dec-3-en-4-yl Ester 2,2-dimethyl-butanoic acid
Structural Formula:	
CAS No.:	148477-71-8
Molecular Weight:	411.4 g/mol
Molecular Formula:	C <sub>21</sub> H <sub>24</sub> Cl <sub>2</sub> O <sub>4</sub>
Batch No.:	NLL5605-7-8
Content(s):	99.1%
Released for Toxicological Investigations until:	August 8th, 1996
Appearance:	white powder
Storage:	at room temperature
Administration:	by diet mixtures in Altromin <sup>®</sup> 1321, containing 1% peanut oil (DAB10)
Frequency of Administration:	twice a week
Preparation:	at room temperature
Storage of Formulation or Diet Mixture:	deep frozen and at room temperature
Formulation(s) Stable over a Period of:	at least 6 days deep frozen and thereafter 4 days at room temperature
Treatment of Controls:	diet mixtures in Altromin <sup>®</sup> 1321, containing 1% peanut oil (DAB10)

#### **4.1 Test Substance and Vehicle**

The test substance was blended (using a mixing granulator manufactured by Loedige, Paderborn) with Altromin<sup>®</sup> 1321 meal. The amount of test substance per concentration was not corrected according to the content of 99.1% active ingredient (see Table 2, page 17).

To all diet mixtures (including 0 ppm) peanut oil (content per kg food: 10 g) was added to minimize dust formation.

#### **4.2 Analytical Examinations of Administration Vehicle**

Data on homogeneity and stability of the test substance in the administration vehicle covering the concentration range used were obtained before the start of this study under the study No. T2060691.

The test substance content of the diet mixtures was checked three times during the study (start of study, end of study, and once in between). This was done by analyzing samples of food mixes used. Per dose one sample of the food mixes was taken on the day the food was prepared and another was taken after being kept under animal housing condition for the maximal feeding period (4 days). All samples taken directly after diet preparation or at the end of the feeding period(s) were kept deep frozen (at temperatures of  $\leq -15^{\circ}\text{C}$ ) until examination.

Reserve samples from each mixture were stored at least for 8 weeks at  $\leq -15^{\circ}\text{C}$ .

### 4.3 Duration of Application, Dosages, Study Groups

The test substance was administered to the animals via the food for the intended period of administration (see Table 1 in Chapter 3, page 14) from the first day of treatment until spontaneous death or scheduled death.

The dose scheme and the distribution of the animals to the groups are given in Table 3 below.

Table 3 - Dosing Schedule

Group No.	Dose (ppm)	Sex	Number of Animals	Animal Number
1	0	m	10	1 - 10
2	100	m	10	11 - 20
3	1000	m	10	21 - 30
4	10000	m	10	31 - 40
5	0	f	10	41 - 50
6	100	f	10	51 - 60
7	1000	f	10	61 - 70
8	10000	f	10	71 - 80

m = males, f = females

In all groups, 10 animals per dose and sex were intended for a subchronic toxicity study with application over the treatment period given in Table 1, page 14.

Before the start of the study, males and females were assigned to the dosage groups. For that purpose the animals were placed singly at the delivery day in cages numbered in ascending order. Thereafter the animal weights were determined and recorded as well as the cage number. Surplus animals with extremely light or heavy body weights were removed. The remaining animals were divided into two weight classes ("light" and "heavy" mice). Using a random list, based on evenly distributed chance numbers especially generated for this study, animals were chosen individually from both collectives and allocated to the group specified by the random list. The animals were placed one after the other in shelves in the order of increasing numbers.

The random lists for this study were produced by the application of the program from the IBM "Scientific Subroutine Package" at the Institute of Biometrics at BAYER AG.

#### 4.4 Rationale for Dose Selection

The dosage scheme for the present study was based on the results of a pilot study (Study No. T4060765; Leser, K.H., unpublished data of BAYER AG, 1996) with **BAJ 2740**.

In this study **BAJ 2740** was administered orally to CD-1 mice (5 males and 5 females per dose) via the diet, in doses of 0- 100- 1000- 10000 ppm over a period of about 4 weeks.

Mortality was not affected by treatment with **BAJ 2740**. Daily observations revealed no abnormalities with regard to body surfaces and orifices, general behavior, posture, respiration and excretory products in any of the animals up to and including doses of 10000 ppm.

In all treatment groups food and water intake as well body weight development were comparable with that of the control animals up to and including 10000 ppm.

Gross pathological investigations in organs and tissues as well as determination of organ weights gave no indication of test-compound-related functional or morphological changes in both sexes up to and including 10000 ppm.

Clinical laboratory and histopathological examinations were not performed.

Under the conditions described the administration of **BAJ 2740** to male and female mice was tolerated without treatment-related lesions up to and including 10000 ppm.

On the basis of these results the following dose scheme was selected for the present subchronic study: 0- 100- 1000- 10000 ppm.

## **4.5 Test System**

### **4.5.1 Experimental Animals**

The study was performed with mice, a species recommended in guidelines for subchronic toxicity studies.

The animals used were SPF-bred CD-1 mice of the strain CrI:CD-1(ICR)BR supplied by the breeder Charles River, Sulzfeld. Animals of this strain are used at BAYER AG since 1995.

The health status of the strain is routinely monitored by random sampling for the most important specific pathogens. The results of these examinations are filed at BAYER AG.

From their arrival until the start of treatment animals for this study were acclimatized to the animal room conditions, during which time their health status was monitored. Only healthy animals, free of clinical signs, were used for the study. The animals were not vaccinated or treated with anti-infectives either before delivery, during the adaptation or study period. Females were nulliparae and not pregnant.

#### 4.5.2 Husbandry

During acclimation and experimental period animals were kept individually, under conventional conditions in Makrolon<sup>®</sup> cages type II (as described by Spiegel and Gönner 1961 and Meister 1965) on low-dust wood granules (supplier: Ssniff Spezialdiäten Inc. Soest/Westfalen; manufacturer: J. Rettenmeier, Ellwangen-Holzühle). Cages and bedding were changed twice weekly.

The wood granules were randomly analyzed for contaminants. Records of these checks are retained on file. Analytic results did not provide any indications of influence on the study objective.

The cages with study animals were kept by groups on shelves in order of increasing animal number. All animals in this study were housed in one animal room in which no other animals were kept.

All the animals were accommodated and housed in a room within a special building domain, separated from other areas by a barrier system. This area could be entered and supplied with materials only through a lock system. For the disposal of used/soiled material and moribund/dead animals there was a separate transport route. The air pressure in the animal rooms was about 20 Pa above the normal pressure. Various additional measures such as changing one's outer clothing, putting on disposable clothing, and disinfecting one's hand and shoes before entering the clean area were aimed to achieve optimal conditions of hygiene.

### Identification of the experimental animals

Identification of the animals was achieved by cards on the cages specifying the test compound, the animal number, dose, sex and study number as well as the corresponding pathology number. In addition, the animals were identified by a tattooed ear number corresponding to the animal number on the cards. The color of the cards for each dose group was different.

### Cleaning, disinfection, pest control

Cages, cage lids, food containers and drinking bottles cleaned with hot water (no detergents or disinfectants) were used during the acclimatization phase and throughout the study. The cage shelves were cleaned routinely with disinfection solution.

Drinking bottles, caps and food containers were replaced regularly. The cage lids and the cage shelves were changed or cleaned once a month.

The floor of the animal room was disinfected once a week (Tegol® 2000). Walls were cleaned regularly in the same way.

A continuous pest control was performed using a non-toxic cockroach trap on pheromone basis. The traps were supplied by Killgerm GmbH, Neuss, Germany. They were placed in the animal room and replaced about every 4 weeks by new ones. The contact of pheromone with experimental animals was avoided in any case.

### Climatic conditions

The animal rooms had a standardized climate:

Room temperature:  $22 \pm 2^\circ\text{C}$

Air humidity:  $55 \pm 5\%$

Light/ Dark cycle: 12 hour rhythm from 6 a.m. to 6 p.m. CET  
(artificial illumination: approx. 140 LUX,  
for work in the room approx. 380 LUX).  
From 6 p.m. to 6 a.m. orientation light,  
approx. 3-5 LUX

Air exchange: approx. 15-20 passages per hour

Occasional deviations from these standards occurred, e.g. during cleaning of the animal room. They did not have any apparent influence on the outcome of the study.

### Nutrition

The diet consisted of a fixed-formula standard diet (Altromin® 1321 meal; supplied by Altromin GmbH, Lage) and tap water during the acclimatization period and throughout the study. Food and water were available for ad libitum consumption.

The nutritional composition and contaminant content of the standard diet were routinely checked and analyzed on a random basis. The tap water complied with drinking water standards in accordance with the Deutsche Trinkwasserverordnung vom 5.12. 90, Bundesgesetzblatt Nr. 66, herausgegeben am 12.12.90, Seite 2612<sup>1</sup>.

The results of the analysis of the food and water are held on file. The data available provided no evidence of any effect on the study objective.

The food was provided in stainless steel containers. Water was supplied in polycarbonate bottles with a capacity of approx. 300 ml (as described by Spiegel and Gönner 1961).

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<sup>1</sup> German Drinking Water Decree of 5.12.90, Federal Legal Gazette No. 66, issued on Dec. 12,1990, page 2612

## 4.6 General Investigations

Table 4 - Frequency and Dates of Determinations

Inspection of Animals:	twice daily, once daily on weekends and public holidays
Determination of :	
Body Weight(s)	weekly
Food Consumption	weekly (over the feeding period of 4 days)
Water Consumption	weekly
Feeding Periods	3 or 4 days
Total Feeding Period	13 weeks
Clinical Laboratory Investigations:	
Hematology	weeks 12/13
Clinical Chemistry	weeks 13/14

#### **4.6.1 Inspection of Experimental Animals**

The experimental animals were inspected at regular intervals given in Table 4. Any clinical signs (findings) and abnormalities were recorded. A detailed weekly report on the condition of the individual animals assessed the following: body surfaces and orifices, posture, general behavior, breathing and excretory products. Findings and abnormalities were recorded on-line both in coded or uncoded form ( $\equiv$  entering free text comments).

#### **4.6.2 Determination of Body Weights**

The body weights of the individual experimental animals were determined and recorded on-line before initial application (week "0" in the tables) and thereafter as indicated in Table 4 on the previous page. Furthermore, body weights were recorded immediately before scheduled necropsies, for calculation of relative organ weights.

#### 4.6.3 Determination of Food, Water and Test Substance Intake

Food intake was calculated for all animals per group individually once a week from the difference of food supplied and not consumed. From these primary data the following were calculated for the period given in Table 4, page 26, if appropriate:

for each interval -

- a) daily food intake per animal
- b) daily food intake per animal and kg body weight
- c) mean daily food intake per animal
- d) mean daily food intake per animal and kg body weight

for the total feeding period -

- e) mean food intake per animal and day
- f) mean food intake per animal kg body weight and day

The calculation of the cumulative data (see below) was based on the period(s) given in the corresponding table(s) in Chapter 5.4, page 41.

- g) cumulative food intake per animal
- h) cumulative food intake per kg body weight and day

Furthermore, from these primary data the following was calculated:

- i) individual test substance intake per kg body weight/day for each specified feeding period
- k) cumulative test substance intake per animal and per kg body weight
- l) mean test substance intake per animal/day and per kg body weight/day for the entire duration of the study

Comparable calculations were done for the water intake of all animals for the period given in Table 4, page 26.

The algorithm used for calculating intake of food and test compound is described in Part 2.

## 4.7 Clinical Laboratory Investigations

Clinical laboratory tests on blood samples were performed on 10 animals per group in the week(s) given in Table 4, page 26.

Occasionally, sample quantity may have been insufficient to permit determination of all intended parameters, or no determination was possible due to technical faults. Therefore, 10 determinations per group are not necessarily available in all cases. The determinations were carried out according to standardized methods (methods and abbreviations used see also Report Part 2), which are subjected to regular internal and external quality control.

Additional comments on appearance of the samples recorded in the raw data for individual cases are not included in the report listings when they were considered to be of no relevance to the corresponding result, i.e. where there was no detectable correlation to treatment.

### 4.7.1 Collection of Samples

The blood samples for determination of glucose concentrations in deproteinized whole blood were taken in the morning from one of the caudal veins of non-fasted, non-anesthetized animals.

The blood samples used for determining the other parameters in peripheral blood were also collected in the morning from the retro-orbital venous plexus of non-fasted animals anesthetized with diethyl ether (Nöller 1955). The blood obtained was treated as follows:

The samples for the hematological determinations were collected in tubes coated with EDTA (anticoagulant).

The samples for the determinations of the thromboplastin time (HQuíck) were collected in tubes with sodium-citrate.

The samples for glucose determinations were mixed with perchloric acid (1:10) to precipitate proteins.

The samples for other biochemical tests were heparinized.

### 4.7.2 Hematology

The following hematological parameters (the abbreviations used in the tables are given in brackets) were determined in peripheral blood:

- Differential blood count
- Erythrocyte morphology (= red blood cell morphology)
- Erythrocyte count (= red blood cell count; ERY)
- Hemoglobin concentration in the blood (HB)
- Hematocrit (= packed cell volume; HCT)
- Leukocyte count (= white blood cell count; LEUCO)
- Mean corpuscular hemoglobin (MCH)
- Mean corpuscular hemoglobin concentration (MCHC)
- Mean corpuscular cell volume (MCV)
- Thrombocyte count (= platelet count; THRO)
- Thromboplastin time (Hepato-Quick; HQUICK)
- Reticulocytes (RETI)

### 4.7.3 Clinical Chemistry

The following parameters (the abbreviations used in the tables are given in brackets) were determined:

#### Enzyme Activities in the Plasma

- Alanine aminotransferase (ALAT)
- Alkaline phosphatase (APh)
- Aspartate aminotransferase (ASAT)

#### Substrates in Plasma

- Albumin (ALB)
- Bilirubin (BILI-t)
- Cholesterol (CHOL)
- Creatinine (CREA)
- Glucose (GLUC)
- Total protein (PROT)
- Triglycerides (TRIGL)
- Urea (UREA)

## **4.8 Necropsies**

All animals scheduled for necropsy were killed by exsanguination under diethyl ether anesthesia, necropsied and their organs and tissues subjected to thorough gross pathological examination. Changes were described in terms of localization, size, color and consistency whenever appropriate.

### **4.8.1 Necropsies on Intercurrent Deaths**

Animals that died spontaneously or were killed in a moribund state during the study were dissected at the earliest opportunity. From these animals the organs and tissues were handled as described in Chapter 4.8.2. Tissues modified by autolysis were fixed only if they were still usable for further histological examination.

#### 4.8.2 Scheduled Necropsies

At the end of the treatment period all surviving animals were necropsied.

The following organs and tissues, in whole or in part, as well as all tissues with macroscopic findings were fixed in 4% formaldehyd solution:

Adrenals	Pancreas
Aorta	Pituitary
Brain (Cerebrum, Cerebellum, Pons/Medulla)	Physical Identifier (tattooed Ears)
Cecum	Prostate
Colon	Rectum (with remaining Intestine)
Duodenum	Salivary Glands
Epididymides	Sciatic Nerve
Esophagus	Seminal Vesicles (with Coagulating Glands)
Eyes (with Eyelids)	Skeletal Muscle
Exorbital Lacrimal Glands	Skin (Mammary Region)
Femur (incl. Bone Marrow and Knee Joint)	Spinal Cord (cervical, thoracal, lumbar)
Gallbladder	Spleen
Harderian Glands	Sternum (with Bone Marrow)
Head-Nose-Pharynx area	Stomach (Forestomach and Glandular Stomach)
Heart	Testes
Ileum	Thymus (if present)
Jejunum	Thyroid (with Parathyroids)
Kidneys	Tongue
Larynx	Trachea
Liver	Ureter
Lungs*	Urethra
Lymph Nodes (mandibular and mesenteric)	Urinary Bladder*
Mammary Glands	Uterus (with Cervix)
Optic Nerves	Vagina
Ovaries (incl. Oviduct)	Zymbal Glands
and all tissues showing abnormalities	

\* prefixation by instillation with 4% formaldehyde

#### 4.9 Organ Weights

The following organs of the animals killed at the end of the treatment were weighed before fixation: brain, liver, spleen, kidneys (both), adrenal glands (both), ovaries (both) and testes (both).

#### 4.10 Histopathological Examinations

The organs and tissues listed in Chapter 4.8, page 32, were handled as follows:

Osseous tissues (femur, head, sternum and vertebrae with spinal cord) were first decalcified with EDTA (ethylene diamine tetraacetic acid tetrasodium salt) and then, like all other organs, embedded in Paraplast.

Sections approximately 5  $\mu$ meter thick were prepared from the organs listed above (except eyelids, larynx, remaining intestinal tissues, ureters, urethra and physical identifier) as needed for histological examination (indicated below) and stained with hematoxylin and eosin (H&E).

Cryocuts obtained from the formaldehyde-fixed livers from all animals were stained with Oil Red O (ORO).

The histopathological examination was limited to the organs/dose groups listed below:

Organs	Dose Groups
Liver, adrenal glands and necropsy findings	all dose groups
Kidneys, testes, epididymides, spleen, thyroid glands and intestine	control + high-dose group

Further details on methodology and scope of microscopic examination are given in Report Part 3.

#### **4.11 Computer-Assisted Data Processing**

The following data were recorded on- or off-line: Results of animal observations and clinical laboratory tests, body, food, water and organ weights. Details on processing of histological data are given in the pathology report.

#### **4.12 Statistics and Presentation of the Results**

The statistical evaluation of data related to clinical chemistry, hematology, body and organ weights as well as feed and water intake is performed using SAS### routines.

Furthermore, in Part 2 of this report all individual quantitative results of the clinical laboratory examinations, the determinations of the animal weights, the food and water intake, and the organ weights, are presented in summary tables showing descriptive analyses as well as in tables showing animal individual data.

In the Section "Results" of Report Part 1 the data are presented in summary tables in form of groups means whereby significant differences from the control group are indicated with "+" for  $p \leq 0.05$  and "++" for  $p \leq 0.01$ .

#### 4.13 Abbreviations used in Report Part 1

##### Miscellaneous

m, M	male
f, w, F	female <sup>2</sup>
n	number
KGW	body weight
sec	seconds
fl	femtoliter
ml	milliliter
mg	milligram
g/l	grams per liter
l/l	liters per liter
pg	picogram
U/l	units per liter
mU/g	milliunits per gram
nmol/g	nanomols per gram
mmol/l	millimols per liter
μmol/l	micromols per liter
mg/mmol	milligrams per millimol
e.g.	for example
No.	number
approx.	approximate
a.m.	in the morning
p.m.	post meridiem
Body W.	Body Weight

##### Statistical data

+	difference against control for $p \leq 0.05$ significant
++	difference against control for $p \leq 0.01$ significant
#	test not applicable due to a low number of samples ( $\equiv 0$ in Tables in Report Part 2)
n.t.	not tested
n.s.	not significantly different from controls
n.c.	not calculated

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<sup>2</sup> for technical reasons the German abbreviation W for female is used in some figures and tables

## 5 RESULTS

The following is a summarized presentation of the results. For the abbreviations used in the tables or figures see preceding page as well as the list of parameters investigated (Chapters 4.7.2 to 4.7.3). The individual values for the statistical calculations are given in the tables in Part 2 of the Report.

### 5.1 Analyses of the Test Substance in the Administration Vehicle

Homogeneity and stability of **BAJ 2740** in the administration vehicle were checked prior to study start. These analytical investigations showed the test substance to be homogeneously distributed and stable in the concentration range used over the period of use (see also Table 2 in Chapter 4, page 17). The results of these investigations are given in Part 2 of the Report.

The content of the test substance in the diet was checked three times during the study. The analytical data verified that the test compound content agreed with the target concentrations within the defined limits (for documentation see Report Part 2).

## **5.2 Inspection of Experimental Animals, Mortality**

If clinical findings were observed during the inspections of the animals the results are presented in Report Part 2 in the form of group incidences and individual animal findings without indication of intensities.

No evidence of treatment-related effects could be deduced.

No animal died during the study. Thus, mortality was unaffected.

## **5.3 Body Weights**

Individual body weights and corresponding group means with statistical data on all groups are given in Part 2 of this report. Figures 1 and 2 , pages 39 and 40, show a plot of the mean body weight development in relation to time for male and female mice.

Tables 5 and 6 present the mean body weight per group and the mean body weight gain per date of determination.

All treated groups did not show marked differences to body weights of controls (the mean differences during the treatment period were in ascending order of dose levels: +0.8%, -2,1% and -2.8% in males and +1.4%, +2.6% and -4.2% in females).

Table 5 - Body Weights (g)

Sex	M	M	M	M	F	F	F	F
Dose (ppm)	0	100	1000	10000	0	100	1000	10000
Week								
0	26.4	27.1	27.0	26.9	23.5	24.1	24.3	23.3
1	28.7	29.5	29.4	29.2	24.4	24.8	25.0	24.0
2	30.2	31.0	31.1	30.9	25.1	25.8	25.8	24.7
3	31.4	32.1	31.9	31.4	25.9	26.2	26.2	24.7
4	32.7	33.3	32.9	32.3	26.2	26.8	27.0	25.0
5	34.0	33.6	33.4	33.4	26.7	27.0	27.6	25.6
6	34.6	34.3	33.6	33.7	27.1	27.2	27.7	25.6
7	35.3	35.3	33.7	34.1	27.1	27.5	28.0	26.0
8	36.4	35.7	34.4	34.3	27.4	27.7	28.0	26.0
9	36.6	36.3	35.1	35.1	28.2	28.4	28.5	27.2
10	37.4	37.7	35.6	34.8	28.4	28.4	29.1	27.1
11	37.2	37.6	35.7	35.4	28.7	29.3	29.4	27.3
12	37.0	37.8	36.1	35.2	28.3	28.9	29.5	26.5
13	36.4	37.2	35.7	35.6	27.8	28.3	28.5	26.8

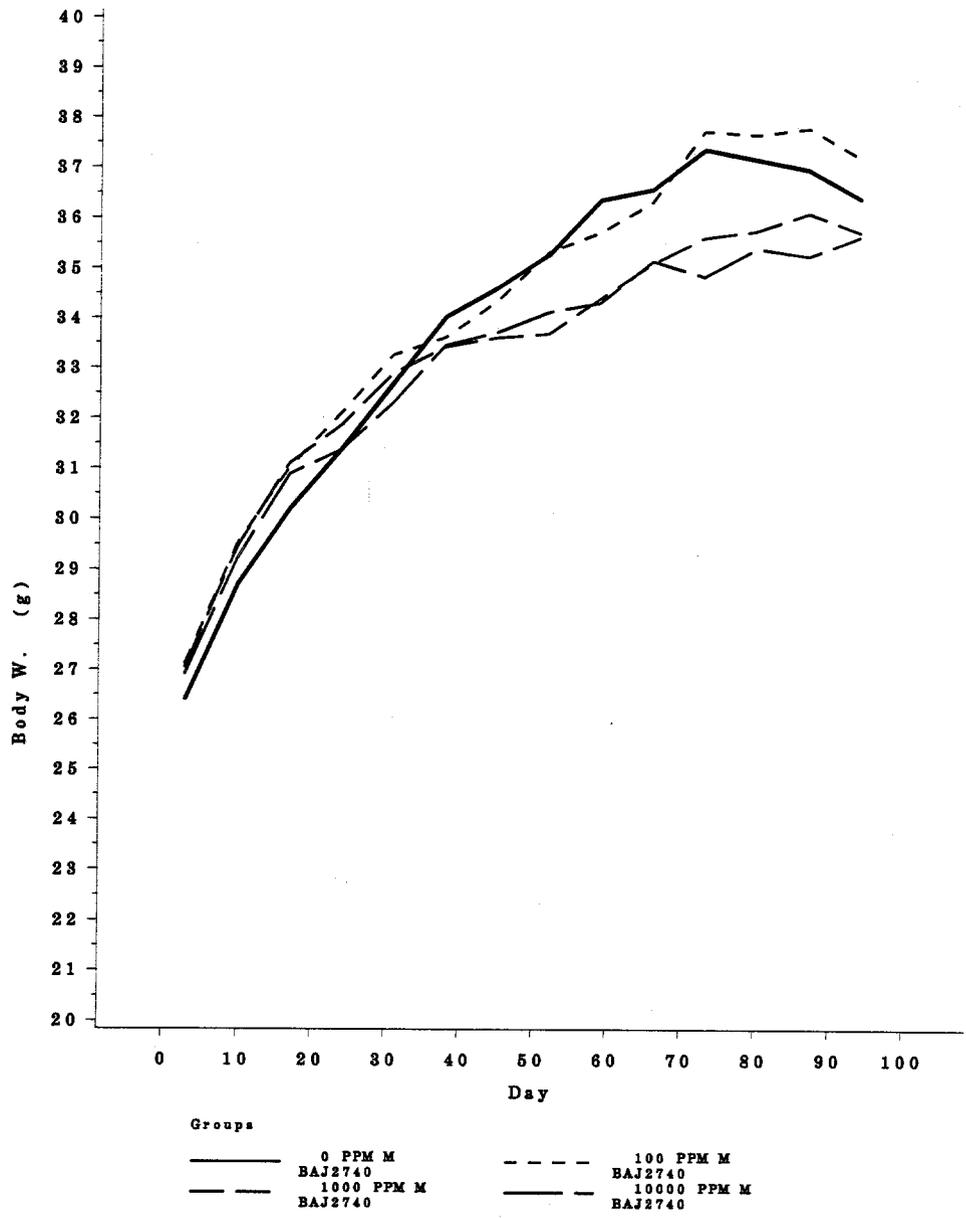
113334/96.001

Table 6 - Body Weight Gain (g)

Sex	M	M	M	M	F	F	F	F
Dose (ppm)	0	100	1000	10000	0	100	1000	10000
Week								
0 - 1	2.3	2.4	2.4	2.3	1.0	0.7	0.7	0.8
1 - 2	1.5	1.5	1.7	1.7	0.7	1.0	0.8	0.7
2 - 3	1.2	1.1	0.8	0.5 +	0.8	0.4	0.3	0.0
3 - 4	1.3	1.1	1.0	0.9	0.3	0.6	0.8	0.3
4 - 5	1.3	0.4 +	0.5	1.1	0.4	0.2	0.7	0.5
5 - 6	0.6	0.7	0.2	0.3	0.4	0.1	0.1	0.1
6 - 7	0.7	1.0	0.1	0.4	0.0	0.3	0.3	0.4
7 - 8	1.1	0.4	0.7	0.2	0.3	0.2	0.0	0.0
8 - 9	0.2	0.6	0.7	0.8	0.8	0.7	0.5	1.1
9 - 10	0.8	1.4	0.5	-0.3 ++	0.2	0.0	0.6	-0.1
10 - 11	-0.2	-0.1	0.1	0.6	0.3	0.9	0.4	0.2
11 - 12	-0.2	0.1	0.4	-0.2	-0.4	-0.3	0.1	-0.8
12 - 13	-0.6	-0.6	-0.4	0.4 ++	-0.5	-0.7	-1.0	0.4

105094/96.001

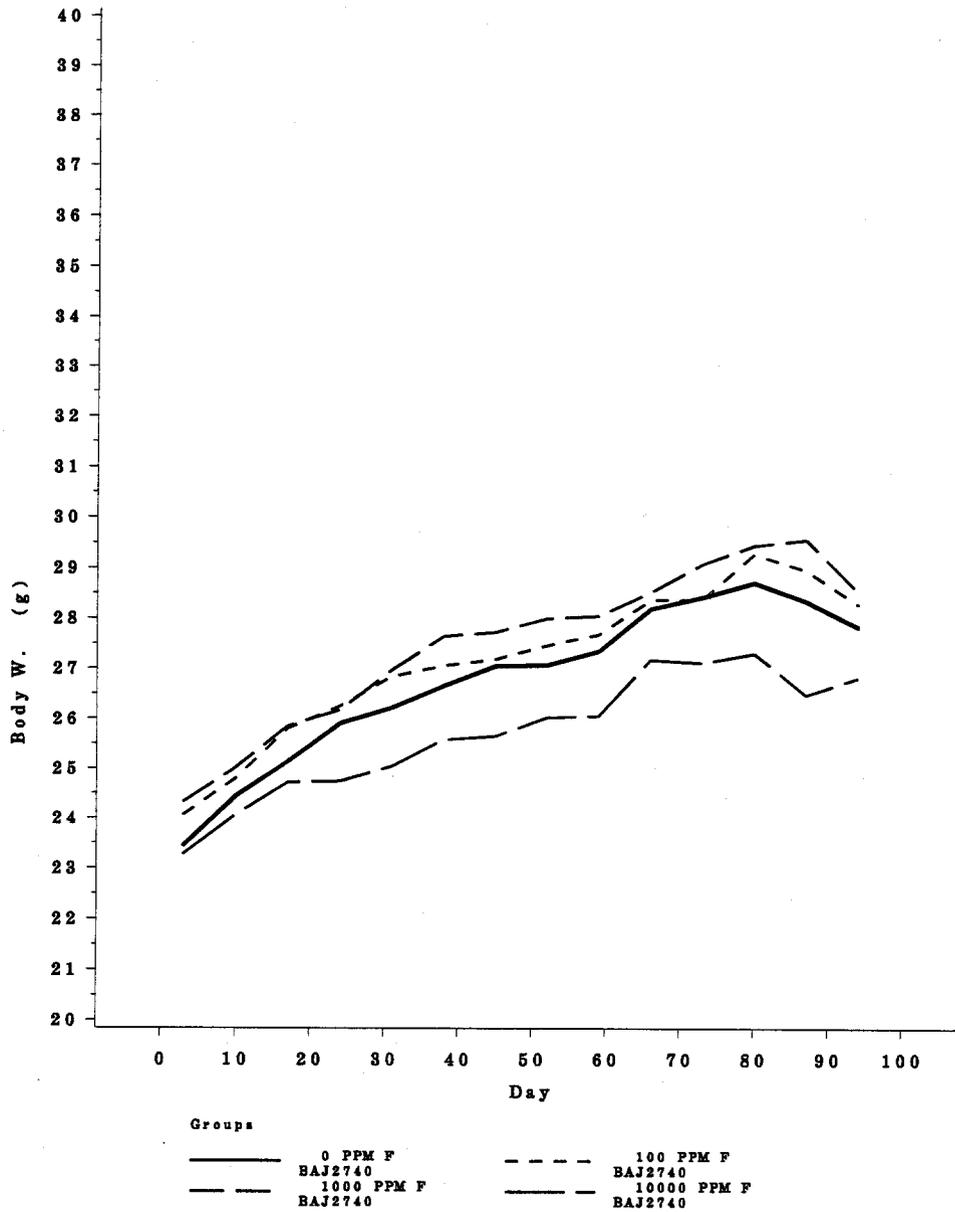
Figure 1 - Body Weights - Males



Study No. T7060885

011314/97.001

Figure 2 - Body Weights - Females



Study No. T7060885

011324/97.001

#### 5.4 Food and Water Consumption, Test Compound Intake

Individual food and water intake data and the corresponding means are documented in Part 2 of this Report. For all groups a survey of mean food, water and test compound intake values and corresponding cumulative values per animal per day and per kg body weight per day is given in Tables 7 to 9.

No toxicologically relevant differences in mean food consumption or water consumption per animal/day or per kg body weight/day were detected in both sexes of all treatment groups.

At all dose levels in either sex the calculations of test compound (**BAJ 2740**) intake revealed comparable data with the selected dose factor of 10.

Table 7 - Food Intake

Cumulative and Mean Food Intake (Group Means)					
Dose ppm	Days	g/animal		g/kg/body weight	
		total	per day	total	per day
<b>Males</b>					
0	91	506	5.6	14814	162.8
100	91	479	5.3	13922	153.0
1000	91	500	5.5	14903	163.8
10000	91	495	5.4	14925	164.0
<b>Females</b>					
0	91	562	6.2	20932	230.0
100	91	756	8.3	27431	301.4
1000	91	588	6.5	21258	233.6
10000	91	627	6.9	24436	268.5

Table 8 - Test Compound Intake

Cumulative and Mean Test Compound Intake (Group Means)					
Dose ppm	Days	g/animal		g/kg/body weight	
		total	per day	total	per day
<b>Males</b>					
100	91	48	0.5	1392	15.3
1000	91	500	5.5	14903	163.8
10000	91	4954	54.4	149252	1640.1
<b>Females</b>					
100	91	76	0.8	2743	30.1
1000	91	588	6.5	21258	233.6
10000	91	6267	68.9	244356	2685.2

Table 9 - Water Intake

Cumulative and Mean Water Intake (Group Means)					
Dose ppm	Days	g/animal		g/kg/body weight	
		total	per day	total	per day
<b>Males</b>					
0	91	606	6.7	17867	196.3
100	91	533	5.9	15487	170.2
1000	91	590	6.5	17548	192.8
10000	91	612	6.7	18416	202.4
<b>Females</b>					
0	91	545	6.0	20312	223.2
100	91	550	6.0	20176	221.7
1000	91	515	5.7	18650	204.9
10000	91	551	6.1	21454	235.8

## **5.5 Clinical Laboratory Examinations**

Clinical laboratory examinations were carried out at the dates given in Table 4, page 26. Individual animal data and group means with statistical information are given in Part 2 of the report.

### **5.5.1 Hematology**

The results of the hematological examinations are presented in the form of arithmetic means in Tables 10 and 11.

In females at 10000 ppm hemoglobin concentration (HB) and packed cell volume (HCT) were slightly lower in comparison to controls. All other parameters examined were unaffected.

There were no treatment related abnormalities in the erythrocyte morphology.

Table 10 - Hematology

Dose ppm	ERY 10E12/l	HB g/l	HCT l/l	MCV fl	MCH pg	MCHC g/l ERY	LEUCO 10E9/l	THRO 10E9/l	HQUICK sec	RETI o/oo
Males - Week 12										
0	8.85	146	0.434	49.3	16.5	337	5.8	1303	18.0	19
100	8.83	145	0.430	48.7	16.5	339	5.9	1325	17.8	18
1000	9.07	147	0.457	50.3	16.2	324	5.4	1345	18.9	18
10000	8.74	142	0.433	49.5	16.2	329	5.3	1290	19.0	17
Females - Week 13										
0	9.02	149	0.449	49.8	16.5	332	3.4	1239	18.9	18
100	9.13	150	0.466	51.1	16.4	322	4.3	1253	19.4	17
1000	9.29	148	0.466	50.2	15.9	318	3.2	1298	19.5	18
10000	8.76	139 ++	0.428	48.8	15.8	325	4.3	1263	19.4	19

105124/96.001

Table 11 - Differential Blood Count

Dose ppm	LYM %	SEGM %	EOS %	MONO %	BASO %	NUCL SH. #/100WBC
Males - Week 12						
0	78.1	19.8	1.1	0.9	0.2	57.0
100	79.9	19.1	1.0	0.0	0.0	68.8
1000	85.6	13.6 +	0.9	0.0	0.0	61.6
10000	84.0	15.2	0.7	0.2	0.0	65.5
Females - Week 13						
0	84.6	14.2	0.9	0.3	0.0	49.2
100	80.8	17.5	1.4	0.4	0.0	54.7
1000	85.7	12.6	1.3	0.3	0.0	42.6
10000	82.3	15.9	1.5	0.4	0.0	45.3

133114/96.001

### 5.5.2 Clinical Chemistry

Tables 12 to 13 present the results of clinical laboratory investigations in the form of group means.

Males and females at 10000 ppm showed slightly lower cholesterol concentrations (CHOL) gaining statistical significance in females.

All other parameters investigated did not show treatment-related effects.

Table 12 - Clinical Chemistry - Enzymes

Dose ppm	ASAT (GOT) U/l	ALAT (GPT) U/l	Aph U/l
Males - Week 13			
0	30.7	29.8	109
100	27.6	27.7	116
1000	30.0	30.8	108
10000	34.0	36.3	117
Females - Week 14			
0	32.4	27.5	141
100	32.1	25.4	154
1000	32.8	31.3	145
10000	33.1	29.8	144

105134/96.001

Table 13 - Clinical Chemistry - Substrates

Dose ppm	PROT g/l	ALBUMIN g/l	GLUC mmol /l	CHOL mmol /l	TRIGL mmol /l	BILI-t mcmol /l	CREA mcmol /l	UREA mmol /l
Males - Week 13								
0	56.0	28.6	6.70	3.44	2.11	1.8	30	11.46
100	56.2	28.4	7.06	3.71	1.97	2.0	28	10.64
1000	55.4	28.4	6.85	3.46	1.19	2.0	28	10.44
10000	56.2	27.8	7.21	2.83	2.49	1.8	32	10.18
Females - Week 14								
0	55.3	31.9	6.15	2.79	1.43	2.1	27	9.76
100	55.4	31.5	6.46	2.53	1.39	2.1	26	9.78
1000	55.5	31.3	6.30	2.59	1.41	2.1	25	9.70
10000	55.4	31.3	6.21	1.93 +	1.90	1.9	27	9.52

105144/96.001

## 5.6 Necropsies

Gross pathological findings in individual animals, the corresponding histopathological findings, and a compilation of the incidences of individual findings are to be found in Report Part 3 (Pathology Report).

For reasons of readability and comprehensibility incidence tables summarize, as far as was possible and sensible, findings of the same nature. No presentation of further details is given (e.g. details of size, color and consistency, or graduations).

Gross pathological examinations revealed no dose-related changes.

## 5.7 Organ Weights

Individual absolute and relative (related to 100 g body weight) organ weights as well as the corresponding group means with statistical information are given in Part 2 of this report. The results are presented as group means in Tables 14 and 15.

At necropsy males at 10000 ppm showed significantly lower absolute and relative kidneys weights.

In males at 1000 ppm absolute and relative liver weights were significantly lower than in controls.

Table 14 - Necropsy - Absolute Organ Weights

Dose ppm	Body W. g	Brain mg	Adrenals mg	Liver mg	Spleen mg	Kidneys mg	Testes mg	Ovaries mg
Males - Terminal Sacrifice								
0	37	514	9	1817	200	618	249	
100	38	496	10	1741	168	582	271	
1000	36	487	8	1606 +	163	580	263	
10000	36	487	8	1780	162	526 ++	271	
Females - Terminal Sacrifice								
0	29	505	12	1402	195	382		39
100	29	511	11	1366	186	414		45
1000	29	512	14	1393	191	406		40
10000	28	510	14	1401	212	364		41

109784/96.001

Table 15 - Necropsy - Relative Organ Weights

Dose ppm	Body W. g	Brain mg /100g	Adrenals mg /100g	Liver mg /100g	Spleen mg /100g	Kidneys mg /100g	Testes mg /100g	Ovaries mg /100g
Males - Terminal Sacrifice								
0	37	1394	23	4907	549	1680	677	
100	38	1315	25	4610	445	1541	719	
1000	36	1350	23	4443 +	451	1605	732	
10000	36	1359	23	4953	453	1463 +	756	
Females - Terminal Sacrifice								
0	29	1765	40	4884	682	1334		136
100	29	1780	40	4761	648	1443		156
1000	29	1767	47	4750	662	1394		134
10000	28	1857	50	5098	768	1327		150

109794/96.001



## 6 DISCUSSION AND EVALUATION

**BAJ 2740** was administered orally to CD-1 mice (10 males and 10 females per dose) via the food, in doses of 0 - 100 - 1000 - 10000 ppm over a period of about 13 weeks. This resulted in a test compound intake of 15.3, 163.8 and 1629.9 mg/kg body weight/day in males and 30.1, 233.6 and 2685.2 mg/kg body weight/day in females.

The animals were regularly observed and weighed and feed and water intake were determined. In addition, clinical laboratory investigations of blood samples were performed. Organs and tissues were subjected to gross and histopathological investigations.

Daily observations revealed no abnormalities with regard to body surfaces and orifices, general behavior, posture, respiration and excretory products in any of the animals up to and including doses of 10000 ppm. Mortality was unaffected by treatment with **BAJ 2740**.

Determination of body weight development did not reveal relevant differences to that of the control animals in mice up to and including 10000 ppm.

No toxicologically relevant differences in mean food and water consumption per animal/day or per kg body weight/day were detected in both sexes of all treatment groups.

In females at 10000 ppm hemoglobin concentration and packed cell volume were slightly lower in comparison to controls. Since all the other hematological investigations in males and females provided no evidence of treatment-related effects on the red and white blood cell counts or of any damage to the hemopoietic organs/tissues the slight changes in females are considered to be incidental and of no toxicological relevance.

Clinical laboratory tests revealed slightly lower cholesterol concentrations in males and females at 10000 ppm which were significantly different from controls in female mice. Histopathology revealed centrilobular hepatocellular hypertrophy in males at 100 ppm and above and in females at 10000 ppm. Periportal cytoplasmic vacuolation was found in 1 male and 3 females at 10000 ppm.

A cytoplasmic vacuolation of the adrenal cortex occurred in males at 10000 ppm and in females at 1000 ppm and above. In 9/10 females at 10000 ppm this alteration was increased in severity, frequently leading to a degeneration of cortical cells accompanied by mononuclear infiltrates.

Both, the alterations in the liver and the adrenal tissues are most likely related to the putative mode of action of **BAJ 2740**. The inhibition of HMG CoA reductase (e.g. by mevinoxin, Goodman et al. 1985; see also Stryer 1985) interferes with the metabolic pathways of cholesterol and steroid synthesis.

At necropsy males at 10000 ppm showed lower absolute and relative kidney weights. In males at 1000 ppm absolute and relative liver weights were lower than in controls. Since clinical laboratory and histological investigations did not yield correlated findings the changes in kidney and liver weights are considered to be of no toxicological relevance.

Gross and histopathological investigations into other organs and tissues gave no indication of test-compound-related functional or morphological changes in both sexes.

Under the conditions described the administration of **BAJ 2740** was tolerated in female mice without treatment-related lesions at 100 ppm, for males there was no no-effect level due to hepatocellular hypertrophy. On the basis of the results obtained from this study the following dose levels were proposed for the subsequent oncogenicity study in mice: 0- 56- 280- 1400- 7000 ppm.

**7 LITERATURE**

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**BAJ 2740**

**Study on Subchronic Toxicity in CD-1-Mice  
(Administration in the Food over 13 weeks)**

**by**

**Leser, K.H., Romeike, A**

**Study-No. T7060885**

**Part 2 of 3**

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**ABBREVIATIONS, METHODS AND REFERENCE VALUES****1 ABBREVIATIONS**miscellaneous

STUDY-NO.	study number
ANIM.NO.	animal number
BODY-W	body weight
W	week
m	male
f	female
n	number
o.B.	without findings
KGW	body weight
p.o., PO	oral
sec	seconds
fl	femtoliter
ml	milliliter
mg	milligram
g/l	grams per liter
l/l	liters per liter
pg	picogram
U/l	units per liter
nmol/g	nanomols per gram
mmol/l	millimols per liter
mcmol/l	micromols per liter
mg/mmol	milligrams per millimol
nmol/g * min	nanograms per gram and minute
μmol/g * min	micromols per gram and minute
Appl. / adm.	administration
g/d	grams per day
Nucl Sh.	nucleus shadow
#/100WBC	number per hundred white blood cells

Statistical data

M	Mean
Med	Median
Min	lowest value in the group
Max	highest value in the group
S.D.	standard deviation
N	number of values/samples in the group
+/*	difference against control for $p \leq 0.05$ significant
++/**	difference against control for $p \leq 0.01$ significant
TS 1%	test result at the $\alpha = 1\%$ significance level
TS 5%	test result at the $\alpha = 5\%$ significance level
0	test not applicable
n.t.	not tested

**2 METHODS OF CLINICAL LABORATORY INVESTIGATIONS**

*Institut für Toxikologie*

*Clinical Laboratories*

**LIST OF METHODS FOR  
CLINICOCHEMICAL AND HEMATOLOGICAL  
EXAMINATIONS**

**Assays in the Laboratories of Clinical Chemistry and Hematology**

The following review lists the routine methods which are used for the assays carried out in the Laboratories of Clinical Chemistry and Hematology.

If a method changed during 1991 and 1992 both references are reported.

Within each section the parameters are in alphabetical order of the short name in English, which is used in the final reports.

## LIST OF METHODS

### Hematological Investigations

DIFF Differential blood count, modified stain according to Wright, "A RAPID METHOD FOR THE DIFFERENTIAL STAINING OF BLOOD FILMS AND MALARIAL PARASITES", Wright, J.H., J. Med. Res. 7, 138 - 144 (1902). Staining with the Hematrak System, Messrs. Beckman, or with the Hema Tek Slide Stainer, Messrs. Miles, Ames Division and manual counting with the microscope.

### Differential blood count

#### Code for nucleus shadows

numbers of nucleus shadows / 100 leucocytes

-		- 5
1	slight	6-10
2	moderate	11-30
3	marked	>30

### Parameter: Norm Ery

By classifying the white blood cells the morphology of the erythrocytes will be determined too.

Norm Ery 1: normal morphology of the erythrocytes

Norm Ery 0: one or more abnormalities have been found.

Code for the red cell morphology, the toxic granulation and the hypersegmented neutrophils.

not reported or blank - no observed abnormality

- 0 one of three examinations showed slight abnormalities
- 1 slight
- 2 moderate
- 3 marked

Code for basophilic stippling

- 0 - 1 basophilic stippled erythrocyte in 10 fields
- 1 2 - 5 basophilic stippled erythrocytes in 10 fields
- 2 1 - 2 basophilic stippled erythrocytes in each field
- 3 > 2 basophilic stippled erythrocytes in each field

**ERY** Erythrocytes - electrical resistance pulse detection with the Sysmex Hematology System from TOA Medical, distribution Messrs. Sysmex, Norderstedt.

**HB** Hemoglobin - measurement with the Sysmex Hematology System from TOA Medical, distribution Messrs. Sysmex, Norderstedt, using sulfolyzer - reagent or alternatively cyanid - reagent.

**HCT** Hematocrit - Cumulative pulse height detection with the Sysmex Hematology System from TOA Medical, distribution Messrs. Sysmex, Norderstedt or determination with the Microhematocrit Centrifuge from Messrs. Heraeus Christ

- LEUCO** Leucocytes - Electrical resistance pulse detection with the Sysmex Hematology System from TOA Medical distribution Messrs. Sysmex, Norderstedt For special purposes counting with a chamber. Staining with TÜRK's solution (acetic acid and gentian violet) from Messrs. Merck Darmstadt and counting in the Neubauer - chamber.
- MCH** MCH - Mean Corpuscular Hemoglobin, computed from RBC and HB.
- MCHC** MCHC - Mean Corpuscular Hemoglobin Concentration, computed from HCT and HB.
- MCV** MCV - Mean Corpuscular Volume, computed from RBC and HCT
- RETI** Reticulocytes - Counting with the flow cytometric reticulocyte analyzer Sysmex R 1000 according to Tichelli, A. et al., "Evaluation of the Sysmex R-1000", Americ. Journ. Clin. Path. Vol. 93, 70-78 (1990)
- THRO** Platelet Count - electrical resistance pulse detection with the Sysmex Hematology System from TOA Medical, distribution Messrs. Sysmex, Norderstedt. For special purposes counting with a chamber. Preparation with Thrombo-Plus from Messrs. Sarstedt Art.No. 51334, counting in the Neubauer - chamber

### Clinicochemical Investigations in SERUM, PLASMA, BLOOD

#### **Hemostaseology**

- HQUICK** Hepato - Quick - according to Heene, D.L., "Kontrolle der oralen Antikoagulantientherapie anhand des Hepato Quick-Tests"; Med. Welt 25, 1529 - 1531 (1974)

**Enzymes**

- ALAT      ALAT optimized (Alanin aminotransferase EC 2.6.1.2) -  
GPT      Empfehlungen der Deutschen Gesellschaft für Klinische Chemie\*,  
Z. Klin. Chem. u. Klin. Biochemie, 10, 182 -192 (1972)
- APh      Alkaline phosphatase optimized - (EC 3.1.3.1) - Empfehlungen der  
Deutschen Gesellschaft für Klinische Chemie\*, Z. Klin. Chem. u. Klin.  
Biochemie, 10, 182 - 192 (1972)
- ASAT      ASAT optimized (Aspartat aminotransferase EC 2.6.1.1) -  
GOT      Empfehlungen der Deutschen Gesellschaft für Klinische Chemie\*,  
Z. Klin. Chem. u. Klin. Biochemie, 10, 182 - 192 (1972)

**Substrates/ Electrolytes**

- BILI-t      Bilirubin, total - according to Wahlefeld, A.W., Herz, G. and Bernt, E.,  
"Modification of the Malloy - Evelyn - method for a simple, reliable  
determination of total bilirubin in serum", Scand. J. Clin. Lab. Invest.  
Vol. 29, Suppl. 126, Abstract 11.12 (1972).
- CHOL      Cholesterol enzymatic CHOD - PAP - according to Siedel, J., Hägele,  
E.O., Ziegenhorn, J. and Wahlefeld, A.W., "Reagent for the Enzymatic  
Determination of Serum Total Cholesterol with Improved Lipolytic  
Efficiency", Clin. Chem. 29, 1075 - 1080 (1983)
- CREA      Creatinine, Jaffé, kinetic - modified according to Bartels, H. et al.,  
"SERUM KREATININBESTIMMUNG OHNE ENTEIWEISSEN", Clin.  
Chim. Acta 37, 193 - 197 (1972)

\* Recommendations of the German Society for Clinical Chemistry

- GLUC        Glucose - according to Schmidt, F.H., "Die enzymatische Bestimmung von Glucose und Fructose nebeneinander", *Klin. Wschr.* 39, 1244 - 1247 (1961)
- TRIGL      Triglycerides - enzymatic colorimetric test, modified according to Wahlefeld, A.W., "Triglyceride", in: Bergmeyer, H.U., *Methoden der enzymatischen Analyse*, 3rd Edition, Vol. II, p. 1878- 1882, Verlag Chemie, Weinheim 1974, and according to Trinder, P., "Determination of Glucose in Blood using Glucose Oxidase with an alternative oxygen acceptor", *Ann. Clin. Biochem.* 6, 24 - 27 (1969)
- UREA        Urea, enzymatic UV test - according to Gutmann , I., Bergmeyer, H.U.,  
HST         "Bestimmung von Harnstoff, Glutamat - Dehydrogenase als Indikator-enzym", in: Bergmeyer, H.U., *Methoden der enzymatischen Analyse*, 3rd Edition, Vol. II, 1842 - 1846, Verlag Chemie, Weinheim 1974.

### Proteindiagnostic

- ALB         Albumin - according to Doumas, B.T. et al., "ALBUMIN STANDARDS AND THE MEASUREMENT OF SERUM ALBUMIN WITH BROMCRESOL GREEN", *Clin. Chim. Acta* 31, 87-96 (1971)
- PROT        Total Protein - Biuret method - according to Weichselbaum, T.E., "AN ACCURATE AND RAPID METHOD FOR THE DETERMINATION OF PROTEINS IN SMALL AMOUNTS OF BLOOD SERUM AND PLASMA", *Amer. J. Clin. Path.* 10, 40 - 49 (1946)

## 3 REFERENCE VALUES

Bayer AG / Pharma				Clinical Pathology						MOUSE
Breed: CRL:CD 1				Reference values 1995 - 1996						
Hematology				Sampling: Retroorbital veinplexus, not fasted						
Parameter	Age Week	Sex	N	Mean	Std.Dev	Range		Range		Unit
						- 2s	+ 2s	- 3s	+ 3s	
ERY	11- 20	M	50	9.03	0.580	7.87	10.19	7.29	10.77	10E12/l
ERY	11- 20	F	49	9.35	0.472	8.41	10.30	7.94	10.77	10E12/l
HB	11- 20	M	50	144	8.1	128	160	119	168	g/l
HB	11- 20	F	49	147	4.9	137	157	132	161	g/l
HCT	11- 20	M	50	0.435	0.0316	0.371	0.498	0.340	0.529	l/l
HCT	11- 20	F	49	0.451	0.0305	0.390	0.513	0.360	0.543	l/l
HQUICK	11- 20	M	10	18.0	0.61	16.8	19.2	16.2	19.8	sec
HQUICK	11- 20	F	10	18.9	0.76	17.4	20.4	16.6	21.2	sec
LEUCO	11- 20	M	50	7.0	2.04	2.9	11.1	0.9	13.1	10E9/l
LEUCO	11- 20	F	49	5.3	2.45	0.4	10.2	up to	12.6	10E9/l
MCH	11- 20	M	50	16.0	0.81	14.3	17.6	13.5	18.4	pg
MCH	11- 20	F	49	15.7	0.78	14.2	17.3	13.4	18.0	pg
MCHC	11- 20	M	50	332	22.8	286	378	264	401	g/l ERY
MCHC	11- 20	F	49	326	19.6	287	366	267	385	g/l ERY
MCV	11- 20	M	50	48.2	3.22	41.8	54.6	38.5	57.9	fl
MCV	11- 20	F	49	48.3	3.21	41.9	54.7	38.7	57.9	fl
RETI	11- 20	M	10	19	2.3	14	23	12	25	o/oo
RETI	11- 20	F	10	18	2.5	13	23	10	25	o/oo
THRO	11- 20	M	50	1290	133.6	1022	1557	889	1690	10E9/l
THRO	11- 20	F	49	1156	133.9	888	1424	754	1558	10E9/l
White Blood Cell Differential				Sampling: Retroorbital veinplexus, not fasted						
Parameter	Age Week	Sex	N	Mean	Std.Dev	Range		Range		Unit
						- 2s	+ 2s	- 3s	+ 3s	
LYM	11- 20	M	50	82.4	7.26	68	97	61	100	%
LYM	11- 20	F	49	83.7	6.54	71	97	64	100	%
SEGM	11- 20	M	50	15.7	6.40	3	29	up to	35	%
SEGM	11- 20	F	49	14.7	6.34	2	27	up to	34	%
EOS	11- 20	M	50	1.0	0.99	up to	3	up to	4	%
EOS	11- 20	F	49	1.2	1.02	up to	3	up to	4	%
MONO	11- 20	M	50	0.8	1.44	up to	4	up to	5	%
MONO	11- 20	F	49	0.5	0.86	up to	2	up to	3	%
BAND	11- 20	M	50	0.0	0.00	0	0	0	0	%
BAND	11- 20	F	49	0.0	0.00	0	0	0	0	%
BASO	11- 20	M	50	0.0	0.28	up to	1	up to	1	%
BASO	11- 20	F	49	0.0	0.00	0	0	0	0	%
Nucl.Sh.	11- 20	M	50	64.4	21.8	21	108	up to	130	#/100WBC
Nucl.Sh.	11- 20	F	49	50.0	26.2	up to	102	up to	129	#/100WBC

Bayer AG / Pharma			Clinical Pathology					MOUSE	
Breed: CRL:CD 1			Reference values 1995 - 1996						
Morphology of Erythrocytes/ Table of Incidences			Grading/ Percent						
			0	1	2	3			
Poikilo	11- 20	M	50	96	4	0	0		
Poikilo	11- 20	F	49	98	2	0	0		
Polychr	11- 20	M	50	98	2	0	0		
Polychr	11- 20	F	49	98	2	0	0		

Bayer AG / Pharma			Clinical Pathology					MOUSE		
Breed: CRL:CD 1			Reference values 1995 - 1996							
Clinical Chemistry			Sampling: Retroorbital veinplexus, not fasted							
Parameter	Age Week	Sex	N	Mean	Std.Dev	Range		Range		Unit
						- 2s	+ 2s	- 3s	+ 3s	
ALAT	11- 20	M	49	32.2	8.31	15.5	48.8	7.2	57.1	U/l
ALAT	11- 20	F	46	28.5	7.32	13.9	43.2	6.6	50.5	U/l
ALBUMIN	11- 20	M	20	29.4	2.70	24.0	34.8	21.3	37.5	g/l
ALBUMIN	11- 20	F	20	32.8	2.42	27.9	37.6	25.5	40.0	g/l
Aph	11- 20	M	49	99	29.7	40	159	10	188	U/l
APH	11- 20	F	46	126	31.1	64	188	33	219	U/l
ASAT	11- 20	M	49	30.2	7.39	15.5	45.0	8.1	52.4	U/l
ASAT	11- 20	F	46	34.7	8.63	17.5	52.0	8.8	60.6	U/l
BILI-t	11- 20	M	49	2.2	0.49	1.3	3.2	0.8	3.7	mcmol/l
BILI-t	11- 20	F	46	1.9	0.39	1.1	2.7	0.7	3.1	mcmol/l
CHOL	11- 20	M	49	3.40	0.644	2.11	4.68	1.47	5.33	mmol/l
CHOL	11- 20	F	46	2.51	0.467	1.57	3.44	1.11	3.91	mmol/l
CREA	11- 20	M	49	29	4.5	20	38	15	42	mcmol/l
CREA	11- 20	F	46	28	2.8	23	34	20	36	mcmol/l
PROT	11- 20	M	49	56.0	3.34	49.3	62.7	46.0	66.1	g/l
PROT	11- 20	F	46	55.9	3.64	48.6	63.1	45.0	66.8	g/l
TRIGL	11- 20	M	49	2.33	0.847	0.63	4.02	up to	4.87	mmol/l
TRIGL	11- 20	F	46	1.60	0.584	0.43	2.76	up to	3.35	mmol/l
UREA	11- 20	M	49	11.80	2.244	7.31	16.29	5.07	18.53	mmol/l
UREA	11- 20	F	46	10.45	2.318	5.81	15.09	3.49	17.40	mmol/l
Clinical Chemistry			Sampling: Vene caudalis, not fasted							
Parameter	Age Week	Sex	N	Mean	Std.Dev	Range		Range		Unit
						- 2s	+ 2s	- 3s	+ 3s	
GLUCOSE	11- 20	M	39	6.46	1.278	3.91	9.02	2.63	10.29	mmol/l
GLUCOSE	11- 20	F	27	6.08	0.911	4.25	7.90	3.34	8.81	mmol/l

**CERTIFICATE OF APPROVAL OF THE TEST SUBSTANCE**

**ANALYTICAL INVESTIGATIONS IN THE ADMINISTRATION VEHICLE**

**BAYER AG  
DEPARTMENT OF TOXICOLOGY  
FRIEDRICH-EBERT-STR. 217-333  
D-42096 WUPPERTAL**

**BAJ 2740**

**Analytical Method Validation  
Homogeneity, Stability Data, and Dose Verification  
in Animal Ration**

**ANALYTICAL REPORT**

**Dr. W. Rüngeler**

**Study-No.: T7060885**

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## 2 ABSTRACT

A liquid chromatographic method for the quantification of BAJ 2740 in animal ration was developed. This work was conducted for tests on stability, homogeneity, and for verification of nominal concentration of this test compound in these mixtures. The method, its validation, and the analytical results are presented in this report.

BAJ 2740 was extracted from animal ration with acetonitrile. It was analyzed on a reversed phase (C18) column and 215 nm ultraviolet (UV) detection.

Recovery of BAJ 2740 from rodent ration fortified with 100 or 20000 ppm was ranged 89.0% and 97.0% respectively with a mean recovery of 93.0 %. The limit of quantification for BAJ 2740 is approximately 1.28 µg/ml. Under the conditions of this method, the limit of reliable measurement of BAJ 2740 was approximately 20 ppm for rodent ration.

**The analytical data verify that the test material in the animal ration was homogeneously distributed within the concentration range of 100 ppm to 20000 ppm. Under current sample preparation and handling conditions comparable to those in the actual study the chemical stability was assured for a period of at least 4 days.**

### 3 INTRODUCTION

A liquid chromatographic method for the quantifying of BAJ 2740 in animal ration was developed. This work was conducted for tests on stability, homogeneity, and for verification of nominal concentration of this test compound in these mixtures. The method, its validation, and the analytical results are presented in this report.

BAJ 2740 was blended with Altromin 1321 which was mixed with 1% peanut oil (DAB 10). For analytical investigations, representative samples, produced under the study director's responsibility, were taken at different points of time from the animal feed containing the test compound. These samples were extracted and diluted with acetonitrile and after filtration subsequently quantified by high-performance liquid chromatography (HPLC) with UV-detection (DAD; wavelength: 215 nm). Standard solutions of approved BAJ 2740 were used as a basis for evaluation.

For the analytical quantification of the test compound a concentration range of 1.28 to 128.40 µg/ml was covered. The calibration curve, produced from standard solutions, was prepared anew for each analytical sequence. No independent reference standard was used. The linearity of the calibration curve, however, must be given. Essentially, all sample concentrations were always within the calibration range documented for each sample sequence.

The experimental standard of this part of the study conforms to the OECD Principles of Good Laboratory Practice (GLP) and to the Principles of Good Laboratory Practice (GLP) according to Annex 1 ChemG.

Investigations necessary for drafting the analytical method and performing analyses were conducted in July/August 1996 at the Department of Industrial Toxicology, Institute of Toxicology of Bayer AG, D-42096 Wuppertal-Elberfeld, Friedrich-Ebert-Strasse 217-333.

The protocol, raw data, and the final report are archived in locations specified by Bayer AG, in accordance with GLP requirements.

**Study-No.: T7060885**

The analytical method, its validation (HPLC), and the analytical raw data documentation (homogeneity, stability) was presented in the study No. T2060691.

**4 RESPONSIBLE PERSONS**

Head of the Department of Industrial Toxicology: ..... Prof. E. Löser

Head of Occupational Toxicology: ..... Dr. J. Pauluhn

Analysis: ..... Dr. W. Rüngeler

**5 MATERIALS AND METHOD****5.1 TEST SUBSTANCE**

Test material:	<b>BAJ 2740</b>
Batch No.:	NLL 5605-7-8
Assay for use:	99.1%
Origin of sample:	Bayer AG, Leverkusen
Stability approved until:	Jan. 26, 1997
Appearance:	white powder
Test material storage:	room temperature
Stability of analytical samples:	was ensured throughout the test period
Toxicology feed mixtures:	Altromin 1321 mixed with 1% peanut oil DAB 10

## 5.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

### 5.2.1 Instrument

Extraction: PTFE column  
 High performance liquid chromatograph HP1090 equipped with \*\*  
 - Autosampler  
 - DAD (Diode array Detector)  
 - Integration: HP 3365 DOS-WorkStation/ChemServer \*\*  
 supplied by Hewlett-Packard Inc.  
 Standard laboratory equipment and glassware  
 {Gas tight} Syringes (25 µl; 100 µl; 250 µl; 10 ml ; Hamilton) \*\*

### 5.2.2 Method

Column: LiChrospher RP 18 5 µm; L = 125 mm; ID = 4 mm; Grom \*\*  
 Oven temperature: 40°C  
 Mobile phase: A: 80% buffer solution  
 B: 20% acetonitrile  
 Flow rate: 1.00 ml/min  
 gradient program: time 1 min --> %B= 30  
 time 6 min --> %B= 70  
 time 16 min --> %B= 80  
 time 16.5 min --> %B= 90  
 Injection volume: 25.0 µl (Autosampler)  
 Detector: wavelength: 215 nm  
 band width (BW): 4 nm  
 reference: 450 nm / 80 nm BW \*\*

\*\* or equivalent

## 5.3 SOLVENTS AND CHEMICALS

Acetonitrile Lichrosolv; Merck No. 30  
 o-Phosphoric acid (o-H<sub>3</sub>PO<sub>4</sub>) 85%; Merck  
 Sodium sulfate; Merck No. 6649  
 Sea sand; Riedel de Haen No. 18649  
 Deionized water (Milli-Q-water), available from Millipore unit, Fa. Millipore  
 Buffer solution: 250 µl o-H<sub>3</sub>PO<sub>4</sub> ad 1000 ml Milli-Q-water

## 6 SAMPLE PREPARATION

The test material - animal feed mixtures were prepared under the study director's responsibility. For sample preparation 10 g of test material were mixed with 10 g of a mixture of sodium sulfate/sea sand (1:1/w:w) and filled in an empty glass/PTFE column. 100 ml of acetonitrile were used for extraction, the extracts were filled in a volumetric flask, diluted, and brought up to volume with acetonitrile. The process of dilution was identical for all samples. These solutions are injected onto the HPLC after appropriate dilution.

## 7 CALIBRATION OF THE ANALYTICAL METHOD

To set-up the calibration series, test material solutions in acetonitrile were prepared with appropriate concentrations. The stability of these solutions was checked at room temperature over a period of 1 day. No decrease in concentration was observed.

The method-specific parameters were adjusted on the HPLC instrument. 25.0  $\mu$ l of each calibration concentration was injected for preparation of the calibration curve.

Measurement wavelength: 215 nm (UV spectrum see fig. 1; presented in study No. T2060691)

Fig. 2 showed a typical chromatogram of these external calibration solutions and additionally a food extract chromatogram. A statistically evaluated calibration curve was shown in Fig. 3. This curve was plotted by the integrator and was based upon the injected concentrations. The calibration line was plotted anew for each analysis sequence, and deviations from this calibration range were therefore possible. All sample concentrations were always within the calibration range documented for each sample sequence. The quantitative evaluation was performed by determination and comparing the peak area of **BAJ 2740** of the analytical solution with the peak areas of the external standard solutions.

Retention time: BAJ 2740 approx. 13.4 min; concentration range: 1.28 to 128.40 µg/ml  
1.28 µg/ml was the limit of quantification of the analytical measurement using this method.

Figure 1: UV-spectrum of BAJ 2740

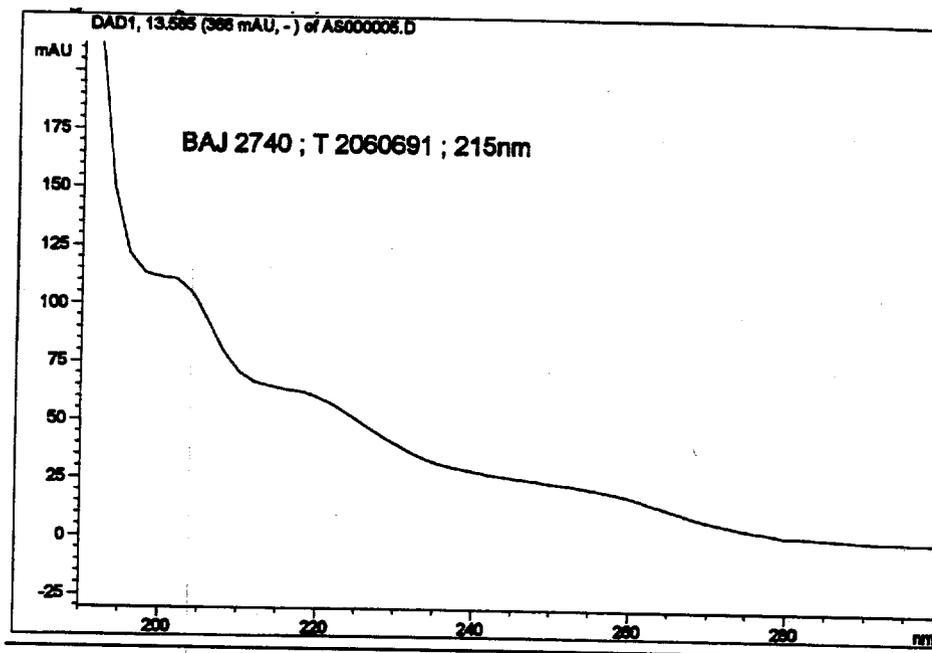


Figure 2.1: typical LC-chromatogram of the test substance as a calibration standard  
test material concentration: 52.44 µg/ml

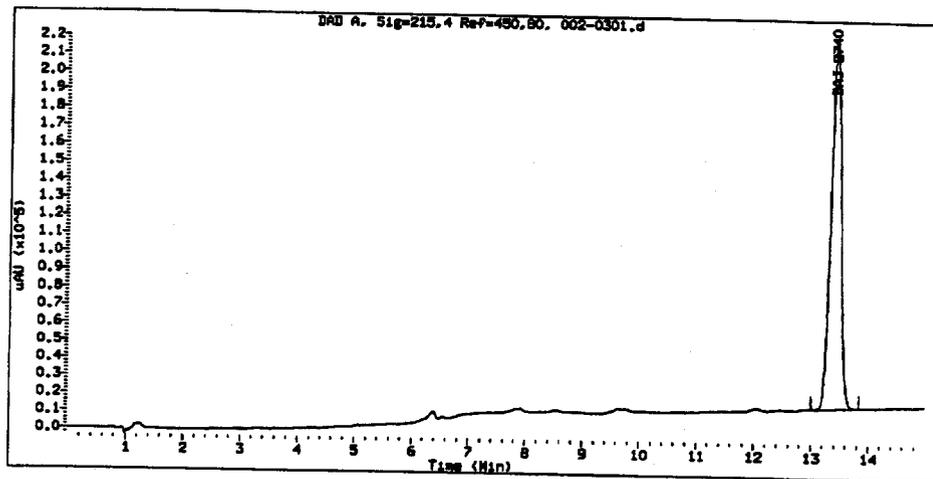


Figure 2.2: typical LC-chromatograms of rodent ration sample:  
test material concentrations: 0 ppm (untreated control sample) and 100 ppm

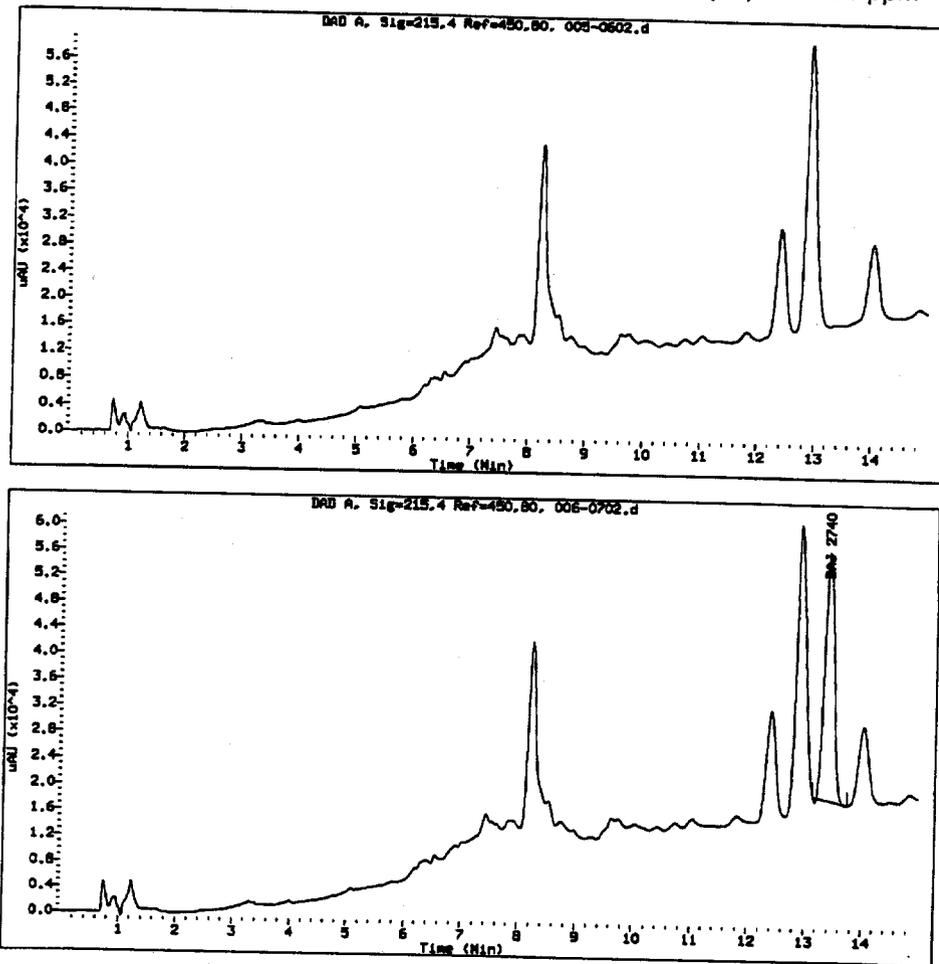


Table 1:

8.340 [ $\mu\text{g/ml}$ ]	128.400 [ $\mu\text{g/ml}$ ]
8.462	129.881
8.492	129.333
8.374	128.983
8.496	129.339
8.416	129.254
8.463	129.689
8.606	129.669
8.471	130.289
8.378	130.368
8.469	130.458
<b>MEAN = 8.463</b>	<b>MEAN = 129.726</b>
<b>%RSD = 0.8%</b>	<b>%RSD = 0.4%</b>

## 9 RECOVERY

The recoveries from rodent ration were evaluated in fortification tests. Known amounts of test compound were added to untreated control feed - Altromin 1321 - prior to extraction.  
Date of preparation: Jan. 23, 1996

### Result:

The analytical data verify that the test material recovery was assured within the concentration range of 100 to 20000 ppm (= 93.0%).

Table 2:

active ingredient added [ppm]	sample	actual-concentration [%] from target	mean value [% ; cv]
20000 ppm	prep.1 -- 1 <sup>st</sup> inj.	102.40	97.01% ; $c_v$ = 8.3%
20000 ppm	prep.1 -- 2 <sup>nd</sup> inj.	101.87	
20000 ppm	prep.2 -- 1 <sup>st</sup> inj.	81.89	
20000 ppm	prep.2 -- 2 <sup>nd</sup> inj.	81.99	
20000 ppm	prep.3 -- 1 <sup>st</sup> inj.	98.76	
20000 ppm	prep.3 -- 2 <sup>nd</sup> inj.	98.62	
20000 ppm	prep.4 -- 1 <sup>st</sup> inj.	100.87	
20000 ppm	prep.4 -- 2 <sup>nd</sup> inj.	100.77	
20000 ppm	prep.5 -- 1 <sup>st</sup> inj.	101.62	
20000 ppm	prep.5 -- 2 <sup>nd</sup> inj.	101.31	

Table 2: (continued)

active ingredient added [ppm]	sample	actual-concentration [%] from target	mean value [% ; cv]
100 ppm	prep.1 -- 1 <sup>st</sup> inj.	92.94	88.99% ; c <sub>v</sub> = 5.4%
100 ppm	prep.1 -- 2 <sup>nd</sup> inj.	92.74	
100 ppm	prep.2 -- 1 <sup>st</sup> inj.	90.39	
100 ppm	prep.2 -- 2 <sup>nd</sup> inj.	91.04	
100 ppm	prep.3 -- 1 <sup>st</sup> inj.	92.00	
100 ppm	prep.3 -- 2 <sup>nd</sup> inj.	91.58	
100 ppm	prep.4 -- 1 <sup>st</sup> inj.	80.23	
100 ppm	prep.4 -- 2 <sup>nd</sup> inj.	80.38	
100 ppm	prep.5 -- 1 <sup>st</sup> inj.	90.33	
100 ppm	prep.5 -- 2 <sup>nd</sup> inj.	88.26	

## 10 ANALYTICAL RESULTS

The analytical data verify that the test material in the animal ration was homogeneously distributed within the concentration range of 100 ppm to 20000 ppm (Tab. 3). Under current sample preparation and handling conditions comparable to those in the actual study the chemical stability was assured for a period of at least 3 days for the 100 ppm ration and 14 days for the 20000 ppm ration (Tab. 4).

An additional stability test was performed with the actual test material batch at all prepared concentrations of the animal ration:

These analytical data verify that the test material content in the toxicology test mixtures agreed with the target concentrations within defined limits. For stability testing the samples were stored for 4/7 days under conditions comparable to those in the actual study, and then quantified. The chemical stability was assured for a period of at least 4 days for the 100 ppm/500 ppm ration and 7 days for the 2500 ppm/ 12500 ppm ration (Tab. 5).

The stability and homogeneity data were presented in study No. T2060691.

At time 0 h the determinations of stability and homogeneity were conducted together at one measurement. For homogeneity assessment samples were taken from different locations (see Table 3.) within the sample containers. The homogeneity and stability data (0 h) were calculated as the average of five separately prepared samples which were injected twice each.

For calculations integrator values from each sample was based on the external standard calibration curve of the active ingredient. The analytical results of the test material were expressed in weight units [ppm]. For assessment of homogeneity and stability the percentage of active ingredient in the original test material was not included for calculations.

## 10.1 HOMOGENEITY

Table 3 presents the analytical results from three samples each collected from a high and low target concentration of rodent feed rations. Each sample was prepared and injected twice.

**Table 3:** Homogeneity tests (Lot No. 1507; 5 kg); date of preparation: Jan. 29, 1996

Sample No.	target concentration		Sample No.
	100 ppm	20000 ppm	
1.1 -- 1 <sup>st</sup> inj.	114.84	21768.63	1.1 -- 1 <sup>st</sup> inj.
1.1 -- 2 <sup>nd</sup> inj.	112.05	21655.69	1.1 -- 2 <sup>nd</sup> inj.
1.2 -- 1 <sup>st</sup> inj.	104.95	22535.74	1.2 -- 1 <sup>st</sup> inj.
1.2 -- 2 <sup>nd</sup> inj.	102.80	22429.02	1.2 -- 2 <sup>nd</sup> inj.
3.1 -- 1 <sup>st</sup> inj.	100.75	21644.60	3.1 -- 1 <sup>st</sup> inj.
3.1 -- 2 <sup>nd</sup> inj.	101.83	21192.54	3.1 -- 2 <sup>nd</sup> inj.
3.2 -- 1 <sup>st</sup> inj.	101.62	22270.68	3.2 -- 1 <sup>st</sup> inj.
3.2 -- 2 <sup>nd</sup> inj.	100.94	21996.91	3.2 -- 2 <sup>nd</sup> inj.
5.1 -- 1 <sup>st</sup> inj.	100.26	22253.13	5.1 -- 1 <sup>st</sup> inj.
5.1 -- 2 <sup>nd</sup> inj.	95.47	21963.90	5.1 -- 2 <sup>nd</sup> inj.
5.2 -- 1 <sup>st</sup> inj.	106.74	22497.44	5.2 -- 1 <sup>st</sup> inj.
5.2 -- 2 <sup>nd</sup> inj.	106.33	22183.27	5.2 -- 2 <sup>nd</sup> inj.
<b>Mean:</b>	<b>104.05 ppm</b>	<b>22032.63</b>	
<b>cv:</b>	<b>cv = 5.1%</b>	<b>cv = 1.9%</b>	

## 10.2 STABILITY (14 Days)

Table 4 presents analytical results from sequential evaluations of two test mixture concentrations. These values were means of two injections from two (three) samples for each concentration per time point. The calculation [%] of target concentrations was based on the analytical result on day 0!

**Table 4: Stability tests (5 kg) in [%] of target concentration and actual weight units [ppm]**  
date of preparation {I} / date of measurement {II}

<b>sample</b>	<b>100 ppm</b> Lot No. 1507	<b>20000 ppm</b> Lot No. 1507
I+II: Jan. 29, 1996 <b>(stab. test-start)</b>	<b>104.1%</b> (104.05 ppm)	<b>110.2%</b> (22032.63 ppm)
I: Jan. 29, 1996 II: Feb. 1, 1996 <b>(stab. 3 days)</b>	<b>87.1%</b> (90.63 ppm) based on day 0	not tested
I: Jan. 29, 1996 II: Feb. 5, 1996 <b>(stab. 7 days)</b>	<b>79.1%</b> (82.33 ppm) based on day 0	<b>96.7%</b> (21295.68 ppm) based on day 0
I: Jan. 29, 1996 II: Feb. 8, 1996 <b>(stab. 10 days)</b>	<b>72.2%</b> (75.10 ppm) based on day 0	not tested
I: Jan. 29, 1996 II: Feb. 12, 1996 <b>(stab. 14 days)</b>	<b>51.4%</b> (53.45 ppm) based on day 0	<b>85.6%</b> (18862.69 ppm) based on day 0

### 10.3 ADDITIONAL STABILITY TEST (7 DAYS)

Table 5 presents analytical results from sequential evaluations of the feed mixture concentrations with frozen reserve samples. These values are means of two individual samples for each concentration. The calculation [%] of target concentration was based on the analytical result on day 0! In analyzed control samples amounts of active ingredient were not detected.

**Table 5:** Content (in [%] of target concentration and actual weight units [ppm])

date of preparation {I} / freezing for stab. testing: see below )\*\*  
samples thawed and date of measurement {II}

sample	100 ppm	500 ppm	2500 ppm	12500 ppm
I: Feb. 21, 1996 II: Feb. 27, 1996 (stab. test-start)	91.5% (91.49 ppm)	95.2% (476.01 ppm)	101.7% (2543.18 ppm)	105.8% (13228.38 ppm)
I: Feb. 21, 1996 II: Feb. 27, 1996 (stab. 4 days)	83.4% (76.33 ppm) based on day 0	87.6% (417.09 ppm) based on day 0	94.9% (2413.19 ppm) based on day 0	101.7% (13455.11 ppm) based on day 0
I: Feb. 21, 1996 II: March 1, 1996 (stab. 7 days)	53.9% (49.32 ppm) based on day 0	71.7% (341.36 ppm) based on day 0	84.1% (2128.71 ppm) based on day 0	104.0% (13758.60 ppm) based on day 0

)\*\*: Samples were prepared on Feb. 21, 1996, frozen to Feb. 23, 1996, stored for 4/7 days, then thawed and quantified.

### 10.4 STABILITY DATA AFTER FREEZING

#### Storage Condition:

After preparation the feeding mixtures were stored 6 days in a refrigerator (approx. <-15°C), thawed (= day 0) and additionally stored for 3 days under conditions comparable to those in the actual study, and then quantified.

#### RESULT:

The analytical data (HPLC) verify that the test material content in the toxicology test mixtures agree with the target concentrations within defined limits (Tab. 6). The additional stability test of these samples was performed after 3 days.

Table 6 presents analytical results from sequential evaluations of the feed mixture concentrations with frozen reserve samples. These values were means of two individual samples for each concentration. The calculation [%] of target concentration was based on the analytical result on day 0! In analyzed control samples amounts of active ingredient were not detected.

**Table 6:** Content (in [%] of target concentration and actual weight units [ppm])  
date of preparation {I} / freezing for stab. testing: see below )\*\*  
samples thawed and date of measurement {II}

sample	100 ppm	500 ppm	2500 ppm	12500 ppm
I: April 10, 1996 II: April 16, 1996 (stab. test-start)	86.7% (86.72 ppm)	98.7% (493.34 ppm)	106.0% (2649.62 ppm)	112.9% (14110.02 ppm)
I: April 10, 1996 II: April 19, 1996 (stab. 3 days)	94.8% (82.23 ppm) based on day 0	96.7% (477.17 ppm) based on day 0	89.8% (2379.03 ppm) based on day 0	95.9% (13530.79 ppm) based on day 0

)\*\*: Samples were prepared on April 10, 1996, frozen to April 16, 1996, stored for 3 days under conditions comparable to those in the actual study. For analytical measurements the samples were thawed and then quantified.

## 10.5 CONTENT CHECKS FOR DOSE VERIFICATION

The analytical data (HPLC) verify that the test material content in the toxicology test mixtures agreed with the target concentrations within defined limits (Tab. 7). Additionally a stability test of these samples was performed. The samples were stored for 4 days under conditions comparable to those in the actual study, and then quantified.

For calculations integrator values from each sample was based on the external standard calibration curve of the active ingredient. The analytical results of the test material were expressed in weight units [ppm]. For assessment of content checks the percentage of active ingredient in the original test material was not included for calculations.

Table 7 presents analytical results from sequential evaluations of the animal rations concentrations. These values were means of two individual samples for each concentration.

The calculation [%] of target concentration was based on the analytical result on day 0!  
In analyzed control samples amounts of active ingredient were not detected.

**Table 7:** Content (in [%] of target concentration and actual weight units [ppm])  
date of preparation {I} / freezing {II}

<b>sample</b>	<b>100 ppm</b>	<b>1000 ppm</b>	<b>10000 ppm</b>
I+II: May 6, 1996 (stab. test-start)	<b>104.9%</b> (104.89 ppm)	<b>100.7%</b> (1007.12 ppm)	<b>98.2%</b> (9820.23 ppm)
I: May 6, 1996 II: May 10, 1996 (stab. 4 days)	<b>85.9%</b> (90.08 ppm) based on day 0	<b>90.0%</b> (906.04 ppm) based on day 0	<b>92.5%</b> (9086.11 ppm) based on day 0
I+II: July 22, 1996 (stab. test-start)	<b>86.6%</b> (86.64 ppm)	<b>102.5%</b> (1025.09 ppm)	<b>108.0%</b> (10794.86 ppm)
I: July 22, 1996 II: **1 (stab. 4 days)	<b>93.4%</b> (80.93 ppm) based on day 0	<b>93.9%</b> (963.02 ppm) based on day 0	<b>100.5%</b> (10849.79 ppm) based on day 0
I+II: July 29, 1996 (stab. test-start)	<b>96.7%</b> (96.67 ppm)	<b>94.4%</b> (944.19 ppm)	<b>108.7%</b> (10868.96 ppm)
I: July 29, 1996 II: **2 (stab. 4 days)	<b>90.8%</b> (87.79 ppm) based on day 0	<b>93.9%</b> (886.66 ppm) based on day 0	<b>97.9%</b> (10641.50 ppm) based on day 0

\*\*1: Samples thawed on July 26, 1996, stored for 4 days under conditions comparable to those in the actual study and then quantified.

\*\*2: Samples thawed on Aug. 2, 1996, stored for 4 days under conditions comparable to those in the actual study and then quantified.

**END OF REPORT**

## SPECIFICATION OF DIET AND DRINKING WATER

NUTRIENT COMPOSITION OF DIET FOR RAT / MOUSE (Altromin 1321 / 1324)

<u>Ingredients *</u>		<u>Amino acids *</u>	
Crude protein	19.0	Lysine	0.90
Crude fat	4.0	Methionine	0.30
Crude fiber	6.0	Cystine	0.30
Ash	7.0	Phenylalanine	0.80
Moisture	13.5	Tyrosine	0.60
Nitrogen-free extract	50.0	Arginine	1.10
		Histidine	0.40
		Tryptophane	0.20
Metabolizable Energy:		Threonine	0.60
Kcal/kg	2850.0	Isoleucine	0.80
Kj/kg	11900.0	Leucine	1.30
		Valine	0.90
<u>Minerals *</u>		<u>Trace elements **</u>	
Calcium	0.9	Manganese	75.0
Phosphorus	0.7	Iron	180.0
Magnesium	0.2	Copper	13.0
Sodium	0.2	Zinc	70.0
Potassium	1.0	Iodine	0.9
		Fluorine	15.0
<u>Vitamins</u>			
<u>Standard-Diet ***</u>			
Vitamin A	15000.0	IU	
Vitamin D3	600.0	IU	
Vitamin E	75.0	mg	
Vitamin K3	3.0	mg	
Vitamin B1	18.0	mg	
Vitamin B2	12.0	mg	
Vitamin B6	9.0	mg	
Vitamin B12	24.0	mcg	
Nicotinic acid	36.0	mg	
Pantothenic acid	21.0	mg	
Folic acid	2.0	mg	
Biotin	60.0	mcg	
Choline	600.0	mg	
Vitamin C	36.0	mg	

\* Average % content in the diet

\*\* Average mg content in 1 kg diet

\*\*\* Additive / 1 kg diet

**CONTAMINANTS IN THE DIET FOR RAT / MOUSE (ALTROMIN 1321 / 1324)**

Contaminant	Detection Limit	Maximum Content	
<u>Mycotoxins</u>			
<b>Aflatoxin</b>			
B1	2 ppb	10 ppb	
B2	2 ppb	5 ppb	
G1	2 ppb	5 ppb	
G2	2 ppb	5 ppb	
<u>Organo Cl-Compounds</u>			
Tecnazene	0.001 mg/kg	not fixed	
HCB (Hexachlorbenzene)	0.001 mg/kg	0.01 mg/kg	
alpha-HCH	0.001 mg/kg	0.02 mg/kg	
beta -HCH	0.001 mg/kg	0.02 mg/kg	
gamma-HCH (Lindane)	0.001 mg/kg	0.10 mg/kg	
delta-HCH	0.001 mg/kg	0.02 mg/kg	
Quintozene	0.001 mg/kg	} 0.01 mg/kg	
Heptachlor	0.001 mg/kg		as
Heptachlorepoxyde	0.003 mg/kg		Heptachlor }
alpha-Chlordane	0.005 mg/kg	0.02 mg/kg	
gamma-Chlordane	0.005 mg/kg	0.02 mg/kg	
alpha-Endosulphane	0.005 mg/kg	0.10 mg/kg	
beta -Endosulphane	0.005 mg/kg	0.10 mg/kg	
Aldrin	0.003 mg/kg	} 0.01 mg/kg	
Dieldrin	0.003 mg/kg		Dieldrin }
Endrin	0.003 mg/kg	} 0.01 mg/kg } } as } 0.05 mg/kg DDT } } }	
o,p-DDE	0.002 mg/kg		
p,p-DDE	0.002 mg/kg		
o,p-DDD	0.002 mg/kg		
o,p-DDT	0.002 mg/kg		
p,p-DDD	0.002 mg/kg		
p,p-DDT	0.002 mg/kg		
Methoxychlor	0.01 mg/kg		not fixed

**CONTAMINANTS IN THE DIET FOR RAT / MOUSE (ALTRONIN 1321 / 1324)**

Contaminant	Detection Limit	Maximum Content
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**Organo-P-Compounds**

Chlorthion	0.01 mg/kg	0.5 mg/kg
Disulfoton	0.005 mg/kg	0.5 mg/kg
Malathion	0.01 mg/kg	1.0 mg/kg
Parathion (-methyl)	0.005 mg/kg	0.5 mg/kg
Parathion (-ethyl)	0.01 mg/kg	0.5 mg/kg
Sulfotep	0.002 mg/kg	0.5 mg/kg
Fenthion	0.005 mg/kg	1.0 mg/kg
Dimethoate	0.005 mg/kg	1.0 mg/kg
Trichlorphon	0.01 mg/kg	1.0 mg/kg
Fenitrothion	0.01 mg/kg	1.0 mg/kg
Bromophos (-methyl)	0.01 mg/kg	1.0 mg/kg
Bromophos (-ethyl)	0.01 mg/kg	1.0 mg/kg
Chlorfenvinphos	0.01 mg/kg	0.5 mg/kg
Pirimiphos (-methyl)	0.01 mg/kg	1.0 mg/kg
Methidathion	0.01 mg/kg	1.0 mg/kg
Ethion	0.01 mg/kg	0.5 mg/kg

**Heavy Metals**

Lead	0.1 mg/kg	1.5 mg/kg
Cadmium	0.01 mg/kg	0.4 mg/kg
Mercury	0.01 mg/kg	0.1 mg/kg
Arsenic	0.2 mg/kg	1.0 mg/kg
Selenium	0.1 mg/kg	0.6 mg/kg
Copper	1.0 mg/kg	not fixed

<b><u>PCB's</u></b>	0.01 mg/kg	0.05 mg/kg
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**Tolerance ranges of analysis:**

Detection Limit	Tolerance
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5 - 100 ppb	+/- 50 % relative
100 - 200 ppb	+/- 50 ppb absolute
above 200 ppb	+/- 25 % relative

**Specification of Tap - Water**  
(according to "Trinkwasser-Verordnung" 12-05-90,  
BGBL No.66 edited 12-12-90, page 2612 - 2629)

Limits of Chemical Substances in Tap-Water

Substance	Limit mg/l	corresponding to approx. mmol/m <sup>3</sup>	calculated as
Arsenic	0.04 *)	0.5	As
Lead	0.04	0.2	Pb
Cadmium	0.005	0.04	Cd
Chromium	0.05	1	Cr
Cyanide	0.05	2	CN <sup>-</sup>
Fluoride	1.5	79	F <sup>-</sup>
Nickel	0.05	0.9	Ni
Nitrate	50	806	NO <sub>3</sub> <sup>-</sup>
Nitrite	0.1	2.2	NO <sub>2</sub> <sup>-</sup>
Mercury	0.001	0.005	Hg
PAH **)	0.0002	0.02	C
Organic Chloride Compounds ***)			
-1,1,1-Trichloroethane	0.025		
Trichloroethene			
Tetrachloroethene			
Dichloromethane			
-Tetrachloromethane	0.003	0.02	CCl <sub>4</sub>
Pesticides and similar compounds			
-per compound	0.0001		
-compounds in total	0.0005		

\*) from January 1, 1996: 0.01 mg/l

\*\*) PAH = Polycyclic Aromatic Hydrocarbons

\*\*\*) from January 1, 1992: Compounds in total 0.01 mg/l  
Tetrachloromethane 0.003 mg/l

Parameters and limits for the evaluation of the quality of drinking water  
(appendix 4 of the "Trinkwasserverordnung")

### I. SENSORY PARAMETERS

Factor	Limit
1 Coloration	0.5 m <sup>-1</sup>
2 Turbidity	1.5 turbidity units / formazin
3 Odour threshold	2 at 12 °C 3 at 25 °C

### II. PHYSICOCHEMICAL PARAMETERS

Parameter	Limit	calculated as
4 Temperature	25 °C	
5 pH	not less than 6.5 not more than 9.5	
6 Conductivity	2000 µS cm <sup>-1</sup> at 25 °C	
7 Oxidizability	5 mg/l	O <sub>2</sub>

### III. LIMITS FOR CHEMICAL SUBSTANCES

Parameter	Limit mg/l	calculated as	Corresponding to approx. mmol/m <sup>3</sup>
8 Aluminium	0.2	Al	7.5
9 Ammonium	0.5	NH <sub>4</sub> <sup>+</sup>	30
10 Iron	0.2	Fe	3.5
11 Potassium	12	K	300
12 Magnesium	50	Mg	2050
13 Manganese	0.05	Mn	0.9
14 Sodium	150	Na	6500
15 Silver	0.01	Ag	0.1
16 Sulphate	240	SO <sub>4</sub> <sup>2-</sup>	2500
17 Surfactants			
a) anionic	0.2	a) Methyleneblue active substances	
b) non-ionic	0.2	b) Bismuth active substances	

## IV. MICROBIOLOGICAL PARAMETERS

Parameter	Volume of sample to be investigated	Maximal tolerated germ titer
Coliforms	100 ml	0
E.coli	100 ml	0
Streptococcus faecalis	100 ml	0
Sulphite reducing clostridium	20 ml	0

Total number of colonies in 1 ml drinking water should not exceed 100  
(incubation temperature  $20 \pm 2$  °C and  $36 \pm 1$  °C).

**ALGORITHMS****1 CALCULATION OF FEED AND WATER CONSUMPTION AND ACTIVE INGREDIENT INTAKE**

The algorithms described below for the feed consumption are also correspondingly applicable to the drinking water consumption. Body weights and the initial and final weights are measured in grams for the calculation.

**A. FEED CONSUMPTION (Individual Animal Determination)****A1. Feed Consumption per Animal per Day**

$$= \frac{H - R}{nT}$$

H = Weight of administered feed (if necessary, plus weight of feed container) at time of weighing (initial weight)

R = Weight of unconsumed feed (if necessary, plus weight of feed container) at time of weighing back (final weight)

nT = Number of days between weighing and weighing back

**A2. Mean Feed Consumption per Animal per Day (Date-Related)**

$$= \frac{\text{Sum of all Values available at a specific Date}}{\text{No of Values}}$$

All feed consumption values existing at a specific date (per animal per day, see A1) are totaled up. This total is divided by the number of values existing at that date.

**A3. Mean Feed Consumption per Animal per Day**

$$= \frac{\text{Sum of all Values}}{\text{No of Values}}$$

All existing feed consumption values (per animal per day, see A1) are totaled up. This total is divided by the number of existing values.

**A4. Cumulative Feed Consumption per Animal**

$$= (\text{Mean Feed Consumption per Animal per Day}) * n \text{ Days}$$

For mean feed consumption per animal per day, see A3. n Days is established from the total number of feed consumption days (see note at end of section).

**A5. Feed Consumption per kg Body Weight per Day**

$$= \frac{\text{Feed Consumption per Animal per Day}}{\text{Body Weight of the Animal}} * 1000$$

For feed consumption per animal per day, see A1. The value that was obtained within the time interval from the day of weighing back (final wt.) to the day of weighing back minus 7 is taken as the basis for the body weight. If no determination of the body weight of the animals within this time interval was planned, the time interval from the day of weighing back to the day of weighing back plus 6 is taken as the basis. If no body weight value is available within either of these two time intervals, no feed consumption is calculated.

**A6. Mean Feed Consumption per kg Body Weight per Day (Date- Related)**

$$= \frac{\text{Sum of all Values available at a specific Date}}{\text{No of Values}}$$

All feed consumption values existing at a specific date (per kg body weight per day, see A5) are totaled up. This total is divided by the number of values existing at that date.

**A7. Mean Feed Consumption per kg Body Weight per Day**

$$= \frac{\text{Sum of all Values}}{\text{No of Values}}$$

All existing feed consumption values (per kg body weight per day, see A5) are totaled up. This total is divided by the number of existing values.

**A8. Cumulative Feed Consumption per kg Body Weight**

$$= (\text{Mean Feed Consumption per kg Body Weight per Day}) * n \text{ Days}$$

For mean feed consumption per kg body weight per day, see A7. n Days is established from the total number of feed consumption days (see note at end of section).

**Note:** Particularly in the case of long-term studies, the number of study days is not identical for all animals of all groups (mortality; necropsy lasting two or more days). In this case, a fixed day is selected for determining the total number of feed consumption days (n Days). The fixed day is the last day of the study week prior to the start of final necropsy.

**A9. ACTIVE INGREDIENT (AI) INTAKE**

The active ingredient (AI) intake is calculated from the feed consumption data by using a "dose factor".

Where: Dose in ppm, Feed consumption in g, AI intake in mg

$$\text{Dose Factor} = \frac{\text{Dose}}{1000}$$

**A10. Mean AI Intake per Animal per Day**

$$= (\text{Mean Feed Consumption per Animal per Day}) \times \text{Dose Factor}$$

For mean feed consumption per animal per day, see A3.

**A11. Cumulative AI Intake per Animal**

$$= (\text{Cumulative Feed Consumption per Animal}) \times \text{Dose Factor}$$

For cumulative feed consumption per animal, see A4.

**A12. AI Intake per kg Body Weight per Day**

$$= (\text{Feed Consumption per kg Body Weight per Day}) \times \text{Dose Factor}$$

For feed consumption per kg body weight per day, see A5.

**A13. Mean AI Intake per kg Body Weight per Day (Date-Related)**

$$= (\text{Mean Feed Consumption per kg Body Weight per Day at a Specific Date}) \\ \times \text{Dose Factor}$$

For mean feed consumption per kg body weight per day at a specific date, see A6.

**A14. Mean AI Intake per kg Body Weight per Day**

$$= (\text{Mean Feed Consumption per kg Body Weight per Day}) \times \text{Dose Factor}$$

For mean feed consumption per kg body weight per day, see A7.

**A15. Cumulative AI Intake per kg Body Weight**

$$= (\text{Cumulative Feed Consumption per kg Body Weight}) \times \text{Dose Factor}$$

For cumulative feed consumption per kg body weight, see A8.

## **2 STATISTICS AND PRESENTATION OF THE RESULTS**

The statistical evaluation of data related to clinical chemistry, hematology, body and organ weights as well as feed and water intake and - if appropriate - survival rate is performed using SAS### routines.

Statistical evaluations on body weight and organ weight data were done using the Dunnett-test in connection with a variance analysis. A Kruskal-Wallis-Test with a Steel-Test was performed when data of feed and water intake were analyzed. The statistical tests used to evaluate the remaining parameters are outlined in a subsequent chapter.

### **A1. Descriptive Analysis**

All variables that are not dichotomous are described by sex, dose group and date using appropriate measures of central tendency (mean, median) and general variability (standard deviation, minimum, maximum).

### **A2. Statistical Tests**

For the statistical evaluation of samples drawn from continuously distributed random variates three types of statistical tests are used, the choice of the test being a function of prior knowledge obtained in former studies. Provided that the variates in question can be considered approximately normally distributed with equal variances across treatments, the Dunnett test is used, if heteroscedasticity appeared more likely a p value adjusted Welch test is applied. If the evidence based on experience with historical data indicates that the assumptions for a parametric analysis of variance cannot be maintained, distribution-free tests in lieu of ANOVA are carried out, i.e. the Kruskal-Wallis test followed by adjusted Mann-Whitney-Wilcoxon tests (U tests) where appropriate.

Global tests including more than two groups are performed by sex and date, i. e. each sex x date level defines a family of tests in the context of multiple comparison procedures (MILLER, R.G., Simultaneous statistical inference, 2nd edition, Springer,

Berlin, Heidelberg, New York, Tokyo, 1981). Within such a family, the experiment wise error is controlled. If not otherwise noted, all pairwise tests are two-sided comparisons. Significant differences from the control group are indicated with "+" for  $p \leq 0.05$  and "++" for  $p \leq 0.01$ .

### **A3. Continuous Random Variables**

Due to the right skewness often encountered in the respective empirical distributions, relative organ weights are submitted to a logarithmic transformation prior to the statistical analysis. Apart from that all variables are analyzed in the raw data form.

#### *Dunnett Test*

The Dunnett test (DUNNETT, C.W., J. Amer. Statist. Ass. 50, 1096, 1955; DUNNETT, C.W., Biometrics 20, 482, 1964; DUNNETT, C.W., J. Amer. Statist. Ass. 75, 789, 1980) compares the outcome of each treatment group with the corresponding control group, regardless of the result of the overall F test (ANOVA). Within this procedure, the designated overall type-one error ### (significance level) is maintained. When applying the Dunnett test it is assumed that the data satisfy the usual ANOVA assumptions, i. e., the data stem from homoscedastic normal distributions.

The calculations are performed with the SAS### procedure PROC GLM.

#### *Adjusted Welch Test*

It is a well known fact that heterogeneous error variances may pose serious problems in statistical inference. Although even in the two-sample case there is no exact solution to the so-called Behrens-Fisher problem (BEHRENS, W.V., Landwirtschaftliche Jahrbücher 68, 807, 1929; FISHER, R.A., Annals of Eugenics 9, 174, 1939), some useful approximations are available. The Welch test (WELCH, B.L., Biometrika 34, 28, 1947) is easy to establish and has several advantages when compared to its competitors (WINER, B.J., Statistical principles in experimental

design, 2nd edition, p. 42, McGraw-Hill, New York, 1971; BEST, D.J., RAYNER, J.C.W., *Technometrics* 29, 205, 1987).

In order to control the family wise type-one error rate within each sex x date constellation Holm's sequentially rejective multiple test procedure is applied (HOLM, S., *Scand. J. Statist.* 6, 65, 1979). For example, in a study comprising one control and k treatment groups the p values are first sorted in increasing order

$$P(1) \leq P(2) \leq \dots \leq P(k)$$

and compared with numbers

$$\frac{\alpha}{k}, \frac{\alpha}{k-1}, \frac{\alpha}{k-2}, \dots, \alpha.$$

The corresponding null hypothesis  $H_j$  is rejected if

$$p^{(j)} \leq \frac{\alpha}{k-j+1},$$

otherwise all  $H_m$  with  $m > j$  are retained without further tests.

The respective calculations are done within SAS###.

### *Kruskal-Wallis Test Followed by Adjusted U Tests*

Nonparametric methods require fewer assumptions about the underlying populations from which the data are obtained. In particular, nonparametric procedures forgo the traditional assumption that the sample consists of realizations of a normally distributed random variable. In addition, they are applicable in situations where not the actual magnitudes of the observations, but rather, their ranks are the target items to be utilized.

In the one-way layout the Kruskal-Wallis test as the nonparametric analogue of the usual parametric univariate analysis of variance is used (KRUSKAL, W.H., WALLIS, W.A., *Amer. Statist. Ass.* 47, 583, 1952). If the resulting p value indicates a nominal significance at the predefined ###-level (0.05 and 0.01, respectively), pairwise treatment control comparisons are performed using the Mann-Whitney-Wilcoxon test

(U test; WILCOXON, F., Biometrics 1, 80, 1945; MANN, H.B., WHITNEY, D.R., Ann. Math. Statist. 18, 50, 1947).

The SAS### procedure PROC NPAR1WAY is used to calculate the test statistics and p values in the asymptotic ###<sup>2</sup>-approximation.

The principle of a sequentially rejective multiple test procedure (that has also been incorporated in the adjusted Welch test) is also applied to the U test for maintaining the family wise type-one error rate.

#### **A4. Discrete Random Variables**

Discrete random variables with more than two possible categories are statistically evaluated using the Kruskal-Wallis test followed by adjusted U tests as outlined under **A2**.

#### **A5. Pathology Data**

Data handling and processing of pathology data which were carried out using the PATHDATA program, version 3.6B, is described in report Part 3.

In this type of statistical processing (**A2** to **A5**) of measurement values a large number of comparisons are made. These multiple tests result in a substantial inflation of the type I error (increase in the number of false-positive statements). However, a comprehensive adjustment taking into account this effect would be unwise, because the false negative rate will rise to possibly unacceptable levels. Thus, ### adjustments are only made within the test families defined above and the p values obtained are considered as additional effect measures rather than clear cut-points distinguishing relevant and irrelevant toxicological effects. On account of this problem, in the evaluation of statistical significance the biological and toxicological relevance is considered.

## **B. Data Presentation in Report Part 2**

In Part 2 of this report all individual quantitative results of the clinical laboratory examinations, the determinations of the animal weights, the food and water intake, and the organ weights, are presented in summary tables showing descriptive analyses as well as in tables showing animal individual data. In the tables, individual values have been rounded up or down. In the calculation of means and variances, etc., the original, unrounded, values were taken as the basis in some cases.

In tables with data on individual body weight determination and food and water intake, occasional values may be missing. Such gaps in primary data and consumption values occur when a measurement value was not recorded as a result of technical error in the on-line processing, or on account of an error in weighing being suppressed as not representable (outside the printing format) or unrealistic (e.g. negative food consumption). In addition, after-weight values were suppressed if the corresponding before-weight values were missing. In tables with individual data on clinical chemistry, isolated values may be missing if the sample amount was not sufficient for determination of all parameters or if the reaction was disturbed and therefore could not be evaluated.

The clinical symptoms (findings) are presented by means of cumulative group incidence and individual animal findings with information on the time of occurrence in question. For reasons of a better overview for main findings, only information on localization is given, without any further details of the findings.

## C. Abbreviations and Statistic Tests

### Hematology

ERY	Erythrocytes Test: Analysis of variance followed by Dunnett test
HB	Hemoglobin Test: Analysis of variance followed by Dunnett test
HCT	Hematocrit Test: Analysis of variance followed by Dunnett test
HQUICK	Hepato Quick Test: Kruskal-Wallis test followed by adjusted U test
LEUCO	Leucocytes Test: Kruskal-Wallis test followed by adjusted U test
MCH	Mean Corpuscular Hemoglobin Test: Analysis of variance followed by Dunnett test
MCHC	Mean Corpuscular Hemoglobin Concentration Test: Analysis of variance followed by Dunnett test
MCV	Mean Corpuscular Volume Erythrocytes Test: Analysis of variance followed by Dunnett test
RETI	Reticulocytes Test: Kruskal-Wallis test followed by adjusted U test
THRO	Thrombocytes Test: Analysis of variance followed by Dunnett test

### Differential Blood Count

BASO	Basophils Test: Kruskal-Wallis test followed by adjusted U test
EOS	Eosinophils Test: Kruskal-Wallis test followed by adjusted U test
Kernsch. Nucl Sh.	Nucleus Shadow Test: Not to be submitted to a statistical Test
LYM	Lymphocytes Test: Kruskal-Wallis test followed by adjusted U test
MONO	Monocytes Test: Kruskal-Wallis test followed by adjusted U test
Norm ERY	Normal ERY Test: Not to be submitted to a statistical Test

Poikilo	Poikilocytosis Test: Not to be submitted to a statistical Test
Polychr	Polychromasia Test: Not to be submitted to a statistical Test
SEGM	Segmented Neutrophils Test: Kruskal-Wallis test followed by adjusted U test

#### Clinical Chemistry - Enzyme

ALAT (GPT)	Alanine aminotransferase Test: Adjusted Welsh test
Aph	Alkaline phosphatase Test: Adjusted Welsh test
ASAT (GOT)	Aspartate aminotransferase Test: Adjusted Welsh test

#### Clinical Chemistry - Substrates

ALBUMIN	Albumin Test: Analysis of variance followed by Dunnett test
BILI-t	Bilirubin total Test: Kruskal-Wallis test followed by adjusted U test
CHOL	Cholesterol Test: Adjusted Welsh test
CREA	Creatinine Test: Analysis of variance followed by Dunnett test
GLUCOSE	Glucose Test: Analysis of variance followed by Dunnett test
PROT	Protein Test: Analysis of variance followed by Dunnett test
TRIGL	Triglycerides Test: Analysis of variance followed by Dunnett test
UREA	Urea Test: Analysis of variance followed by Dunnett test