

Location of study

Life Science Research - Roma Toxicology Centre  
Via Tito Speri 12  
Pomezia (Roma) - Italy

Compliance with Good Laboratory Practice

This study will be conducted in compliance with the principles of GLP as set forth in the OECD guidelines for testing of chemicals and with the GLP regulations of the US FDA.

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Study no. 235-003-009

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(Dr. Alfredo Nuzzata)

EXPERIMENTAL PROCEDURE

1. TEST SUBSTANCE

1.1 Identity

It will be the responsibility of the Client to determine, for each batch of substance, the identity, strength, purity and composition, or other characteristics which appropriately define the test substance, before its use in the study. The determination of the stability of the test substance will also be the Client's responsibility.

1.2 Characteristics

Information supplied by the Client is summarized below:

Identity for reporting	: FDP (Fructose-1,6-diphosphate)
Chemical identity	: Fructose-1,6-diphosphate dehydrated sodium salt
Suitable vehicle	: Isotonic saline
<u>Stability</u>	
Test substance	: 5 years
Test substance in vehicle	: #
<u>Storage conditions</u>	
Test substance	: Ambient temperature, protected from light
Test substance in vehicle	: Ambient temperature, protected from light

The following information refers to the original batch of material supplied for the start of the study. Further batches may be required during the course of the study. Full details of batch usage will be maintained in the formulation records but protocol amendments will not be issued.

Date of receipt at LSR-RTC	: 18.10.89
Batch no.	: 521 B
Purity	: 88.30% (FDP Na3H)
Appearance	: Pale yellow powder

Prior to commencement of treatment, a reserve sample of the test substance will be taken and kept under the storage conditions of the bulk supply. At each batch change and after preparation of the final dose a sample of the test substance will be shipped to the Client for analysis; a duplicate sample will be retained at LSR-RTC. Results of these analyses may be reported to LSR-RTC for inclusion in the final report.

# To be added.

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### 1.3 Method and frequency of dose preparation

For the highest concentration of 220 mg/ml the required amount of test substance will be dissolved in isotonic saline and sterilized by Millipore filtration (0.2 micrometres). The lower concentrations of 110 mg/ml and 55 mg/ml will be prepared by serial dilution of the highest concentration with isotonic saline. The solutions will be prepared daily. The dosage volume required for daily administration will be calculated in advance based on the most recently recorded body weight.

### 1.4 Formulation analysis

Samples of the formulations prepared for all treatment groups will be taken in weeks 1 and 13, deep frozen and sent to the Client for analysis.

## 2. ANIMAL MANAGEMENT

### 2.1 Animal supply and acclimatization

A total of 36 pure-bred Beagle dogs of known lineage (18 males and 18 females) approximately 20 weeks of age and estimated to weigh between 5 and 7.5 kg will be ordered from Allevamento Soprani, S. Polo D'Enza, Reggio Emilia, Italy.

All dogs will be vaccinated by the supplier against hepatitis, leptospirosis and distemper (Candur CEL, Boehringerwerke) at approximately 8 and 12 weeks of age. In order to ensure that the animals are suitable for use on study, the dogs will be selected at the supplier's premises by an LSR-RTC veterinary surgeon. Before delivery to LSR-RTC all dogs will be vaccinated against rabies (Dohyvac i-R, Froom Laboratories, Wisconsin, USA) and parvo virus. On arrival each dog will be assigned a unique stock number which will be tattooed on the ear and stamped on a metal disc attached to a collar around the dogs neck. Even numbers will be assigned to males, odd numbers to females. An acclimatization period of a minimum of three weeks will then be allowed before the start of treatment. This period may be extended if necessary.

### 2.2 Health check

As soon as possible after arrival each animal will be subjected to a detailed examination by a veterinary surgeon. An antiparasitic (LEN, Teknofarma) will be given shortly after arrival and this treatment will be repeated twice at 1 day intervals. This course of treatment will be repeated after 20 days. Any additional veterinary treatment required will be recorded in detail.

Shortly after allocation blood samples will be obtained from the jugular vein of each dog for haematological and biochemical screening (see section 5). Overnight urine samples will also be collected. Any animal with a prolonged prothrombin time will be checked for factor VII deficiency.

### 2.3 Animal husbandry

The animals will be housed inside a limited access dog facility. Temperature and humidity will be monitored daily and these data will be retained in archives. A standby power supply will be automatically brought into operation should the public electricity supply fail. Personnel entering will be required to wear protective clothing. On entry to animal rooms additional protective clothing may be worn dependent on the Hazard Classification assigned to the test substance. Dogs will be individually housed in indoor kennels of approximately 0.9 x 1.0 m floor area with access, when possible, to individual open air pens.

Sawdust used as bedding will be changed daily, during this period dogs will be exercised in the kennel area or confined to the open air pen.

### 2.4 Water, diet and sawdust supply

Drinking water will be supplied ad libitum to each kennel via an automatic "Lixit" valve system or water bottles, except when urine is collected. Each dog will be offered 400g daily of a complete pelleted dog diet (Altromin H, A. Rieper, Bolzano, Italy) at least half an hour before dosing.

There is no information available to indicate that any non-nutrient substance likely to influence the effect of the test substance is present in the drinking water or the diet, or in the sawdust bedding material. Records of analysis of water, diet and bedding are kept on file at LSR-RTC.

### 2.5 Allocation to groups

On the day of allocation (shortly after arrival at LSR-RTC) all the dogs will be weighed and temporarily allocated to groups by computerized stratified randomization to give approximately equal initial group mean body weights. Thereafter, pre-treatment investigations will be carried out on all dogs from the batch supplied for the study, including dogs designated as spare animals in the allocation process described above.

Shortly before the start of treatment the allocation will be reviewed according to the following principles:

- (i) Any dog showing signs of ill health will be rejected.
- (ii) Dogs will be rejected if the pre-treatment investigations listed in sections 4 and 5 reveal abnormalities.
- (iii) On completion of (i) and (ii) any factor VII deficient animals will be rejected.
- (iv) The group mean body weight at the start of treatment should be approximately equal.

The allocation will be adjusted where considered appropriate, using the spare dogs from the initial allocation if necessary. Following this review the allocation will be confirmed and no further adjustments or replacements will take place after the first day of treatment.

Each kennel will be identified by a label, colour-coded according to group as detailed below (Section 3.1) and recording the Study number, animal number and details of treatment. This colour coding will match the appropriate colour-coded formulation container. The animal allocation to kennels will be selected to minimize, as far as possible, any environmental effects.

### 3. TREATMENT

#### 3.1 Treatment, group sizes and identification

Each group will comprise 4 male and 4 female dogs. The group identification and animal numbers assigned to the treatment are summarized below:

<u>Group:</u> <u>Colour code</u>	<u>Level</u>	<u>Treatment</u> (mg/kg/day)+	<u>Concentration</u> (mg/ml)+	<u>Dog numbers</u>	
				<u>M</u> (even)	<u>F</u> (odd)
1: white	Control	0	0	2- 8	1- 7
2: yellow	Low	110	55	10-16	9-15
3: blue	Medium	220	110	18-24	17-23
4: red	High	440	220	26-32	25-31

+ In terms of Fructose-1,6-diphosphate dehydrated sodium salt (FDP Na3H).

The number for each dog will be stamped on a metal disc attached to a collar around the dogs neck.

#### 3.2 Administration of test substance

The test substance will be administered intravenously at an approximate rate of 5-8 ml/min via the left and right cephalic and right saphenous veins, used in rotation (the left saphenous vein will act as a within animal control), at least half an hour after feeding. The dose will be administered to each animal on the basis of the most recently recorded body weight at a dose volume of 2 ml/kg body weight. Control animals will receive the vehicle, at the same dose volume.

#### 3.3 Duration of treatment

All animals will be dosed once a day, seven days a week, for a minimum of 13 consecutive weeks.

4. IN VIVO OBSERVATIONS

Dated and signed records of all activities relating to the day-by-day running and maintenance of the study within the animal unit, as well as to the group observations and examinations outlined in this protocol, will be recorded in the Study Day Book.

Animal room observations may be extended to include other tests and the frequency of any of the specified tests may be changed if required by the Client or if it is necessary to further investigate any unusual findings.

4.1 Clinical signs

All clinical signs will be recorded daily for individual animals. Daily records of the kennels will also be maintained (for vomitus, blood, diarrhoea etc.).

Once per treatment week and whenever possible at seven day intervals, each animal will be subjected to an additional physical examination and any abnormality or clinical sign will be recorded.

Dated and signed records of appearance and change of clinical signs will be maintained on clinical history sheets for individual animals.

4.2 Mortality

Throughout the study, all animals will be checked early in each working day and again in the afternoon to look for dead or moribund animals. At weekends and Public Holidays a similar procedure will be followed except that the final check will be carried out at approximately mid-day. This will allow post-mortem examination to be carried out during the working period of that day.

Severely debilitated animals will be observed carefully. Animals judged to be in extremis will be sacrificed.

Where possible ante mortem blood samples will be taken, a physical examination will be performed, and urine samples will be obtained at necropsy. These will be examined in an attempt to establish the cause of morbidity.

A complete necropsy will be performed in all cases as described in Section 6 below.

4.3 Body weight

Each animal will be weighed three times per week during the first week after arrival and then at weekly intervals throughout the remainder of the acclimatization period, twice weekly up to week 4 of treatment, weekly thereafter, and before necropsy.

More frequent weighings may be instituted for animals displaying certain clinical signs, so that the progress of the observed condition can be monitored.

All routine weighings will be performed before feeding.

Heart rate  
Wave intervals  
Amplitudes

The R:T ratio will be calculated from the amplitude measurements.

5. CLINICAL PATHOLOGY INVESTIGATIONS

Twice before commencement of treatment and during weeks 6 and 13, samples of blood will be withdrawn from the jugular vein of each dog after overnight fasting.

Once before commencement of treatment and during weeks 5 and 13, individual overnight urine samples will be collected from all dogs under conditions of food and water deprivation and faecal samples will also be collected, either from the metabolism cage or from the pen.

The blood samples collected will be divided into tubes containing anticoagulant as follows:

EDTA . . . . . for haematological investigations  
Citrate. . . . . for coagulation tests  
No anticoagulant . . . . . for the biochemical tests

The estimations to be performed on blood and urine samples have been listed below:

5.1 Haematology

Erythrocyte sedimentation rate  
Packed cell volume  
Haemoglobin concentration  
Erythrocyte count  
Reticulocyte count (if there are signs of anaemia)  
Mean cell haemoglobin  
Mean cell volume  
Mean cell haemoglobin concentration  
Total leucocyte count  
Differential leucocyte count - Neutrophils  
  - Lymphocytes  
  - Eosinophils  
  - Basophils  
  - Monocytes

Abnormalities of the blood film  
Platelet count  
Prothrombin time  
Partial thromboplastin time

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4.4 Food consumption

The weight of food consumed by each dog will be recorded daily, with due allowance being made for any food spillage.

4.5 Water consumption

Measurements of the water consumed by each animal will be performed over 3 day periods twice during the pre-treatment period, and over a 3 day period during weeks 1, 6 and 12.

4.6 Veterinary examination

Each animal will be subjected to an examination by a veterinary officer before dosing commences and thereafter at approximately monthly intervals in which particular attention will be paid to:

Teeth and gums  
Mucous membranes and skin  
Ears (external auditory canal)  
Superficial lymph nodes  
Abdomen - including palpation  
External genitalia and mammary glands  
Chest including auscultation of heart and lungs  
Stance - including palpation of limbs  
General behaviour and appearance

The outcome of this examination will be recorded for every animal; any abnormalities detected may be photographed or investigated further.

4.7 Ophthalmoscopy

Both eyes of all animals assigned to the study will be examined just prior to the commencement of treatment by means of an ophthalmoscope approximately 20 minutes after the instillation of 1.0% tropicamide (Visumidriatic, Merck Sharp & Dohme). Where possible, animals with non-resolving lesions will be replaced with spare animals showing no ocular abnormality, from the batch initially ordered for the study. The eyes of all animals in all groups will be re-examined during weeks 6 and 12.

4.8 Electrocardiography

Once before treatment commences electrocardiography tracings will be recorded for all dogs using the three standard limb leads (I, II and III) and the three augmented limb leads (aVR, aVL and aVF). During weeks 1 and 12 further tracings will be obtained, just prior to and after administration (where possible within 15 minutes after dosing). From the tracings obtained the following will be measured:

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5.2 Clinical chemistry

Alkaline phosphatase  
Alanine amino-transferase  
Aspartate amino-transferase  
Urea  
Creatinine  
Glucose  
Bilirubin  
Cholesterol  
Total protein  
Protein electrophoretogram - Albumin  
- Alpha-one globulin  
- Alpha-two globulin  
- Beta globulin  
- Gamma globulin  
- Albumin/Globulin ratio

Sodium  
Potassium  
Calcium  
Chloride

5.3 Urinalysis

Appearance  
Volume  
Specific gravity  
pH  
Protein  
Total reducing substances  
Glucose  
Ketones  
Bilirubin  
Urobilinogen  
Nitrite  
Blood

The sediment, obtained from centrifugation at approximately 3000 rpm for 10 minutes, will be examined microscopically for:

Epithelial cells  
Polymorphonuclear leucocytes  
Erythrocytes  
Crystals  
Spermatozoa and precursors  
Other abnormal components

5.4 Faecal analysis

Faecal occult blood.

6. TERMINAL STUDIES

6.1 Euthanasia

Animals in extremis or sacrificed for humane reasons and those that have completed the scheduled test period will be placed under intravenous barbiturate anaesthesia and killed by rapid exsanguination. All animals, including those found dead, will be subjected to necropsy, supervised by a pathologist, as detailed below:

6.2 Bone marrow

During the necropsy procedure, bone marrow samples will be obtained from a rib from all animals. Smears prepared from these samples will be air dried, fixed in methanol and stained using a May-Grunwald-Giemsa procedure. The smears will be examined for abnormalities and the myeloid/erythroid ratio calculated.

6.3 Necropsy procedure

The clinical history of the animal will be studied and a detailed post mortem examination will be conducted (including examination of the external surface and orifices. Changes will be noted, the requisite organs weighed and the required tissue samples preserved in fixative and processed for histopathological examination (see Sections 6.4 and 6.5).

Representative photographs will be taken of any significant findings, if considered appropriate.

6.4 Organ weights

The following organs from all animals completing the scheduled test period will be dissected free of fat and weighed:

Adrenal glands	Pituitary gland
Brain	Spleen
Heart	Testes
Kidneys	Thyroid and parathyroid glands
Liver	Uterus
Ovaries	

The ratios of organ weight to body weight will be calculated for each animal.

At the discretion of the pathologist, organs may be weighed from animals dying or killed prior to the terminal sacrifice.

6.5 Tissues preserved in fixative

Samples of all the tissues listed below will be preserved in 10% buffered formol-saline (except eyes and optic nerves which will be preserved in Davidson's fixative).

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Abnormalities	Nictitating glands
Adrenal glands	Oesophagus
Aorta	Optic nerves
Bone marrow (from sternum)	Ovaries
Brain	Pancreas
Bronchi	Parathyroid glands
Caecum	Pharynx*
Cervix*	Pituitary gland
Colon	Prostate gland
Duodenum	Rectum
Epididymides	Salivary glands
Eyes	Sciatic nerve
Femur* (including articular surface)	Skeletal muscle
Gall bladder	Skin
Heart	Spinal cord
Ileum	Spleen
Injection sites	Sternum
Jejunum	Stomach
Kidneys	Testes
Larynx*	Thymus (where present)
Liver	Thyroid gland
Lungs	Tongue
Lymph nodes - Mesenteric	Trachea
Lymph nodes - Peribronchial	Urinary bladder
Lymph nodes - Submandibular	Uterus
Mammary area	Vagina*
Nictitating membranes	

\* These tissues will be preserved but not processed further in the first instance.

#### 6.6 Histopathological examination

The tissues required for histopathological examination are listed above.

After dehydration and embedding in paraffin wax, sections of the tissues will be cut at 5 micrometre thickness and stained with haematoxylin and eosin.

The tissues specified above from all animals in all dosage groups will be examined.

#### 6.7 Photomicrography

Representative photographs will be taken of any treatment-related lesions. Other photomicrographs may be taken as required by the Client.

7. STATISTICAL ANALYSIS

Standard deviations will be calculated as considered appropriate. For continuous variables the significance of the differences amongst group means will be assessed by analysis of variance. Differences between each treated group and the control group will be assessed by Student's "t" test using a pooled error variance. Further tests will be used as considered appropriate. Details of all tests used and the data to which they are applied will be included in the final report.

8. AMENDMENTS TO PROTOCOL

It is not intended to make any amendment to this Protocol without authorisation by the Client. However, in the event of difficulty in contacting the Client and/or for humane reasons and/or for the protection of scientific integrity the testing laboratory must retain the right to take independent action.

9. REPORTING

9.1 Interim reports

Any unexpected findings during the course of the study will be reported to the Study Monitor immediately.

9.2 Final report

An APC (advance photocopy) of the final report will be sent to the Client. With the exception of the dated signature of scientists and other professional personnel, the APC will contain all information and data included in the final report. Comments made by the Client may be incorporated into the APC, after which it will be issued as the final report. The final report will include the information and data required by current internationally recognized regulations. Five copies will be supplied.

9.3 Corrections or additions to the final report

Corrections or additions to the approved (i.e. signed) version of the final report will be in the form of an amendment by the Study Director. The amendment will clearly identify that part of the final report that is being added to or corrected and the reasons for the correction or addition, and will be signed and dated by the person responsible.

10. RECORDS AND ARCHIVES

Full records will be maintained of all aspects of study conduct, along with results of all measurements and observations. Prior to final archiving of the study data a full list will be prepared of all records associated with the experiment. All specimens, raw data, records and documentation generated during the course of this study will be retained at LSR-RTC during the study and subsequently for a period of at least five years. The data will not be destroyed without the prior consent of the Client. Some data may be stored on computer readable media, in a manner fully compliant with Good Laboratory Practice.

11. QUALITY ASSURANCE

This study will be subjected to the following quality assurance procedures as laid down in Section 58.35 of the GLP Regulations published by the U.S. Food and Drug Administration.

The protocol will be inspected.

Procedures and data relevant to the study will be inspected at intervals adequate to assure the integrity of the study.

The final report will be reviewed to ensure that it accurately describes the methods and Standard Operating Procedures and that the results accurately reflect the raw data.

Periodic reports on these activities will be made to management and the Study Director.

All raw data pertaining to the study will be available for inspection by the study monitor (for scientific monitoring) or the Quality Assurance Unit of the Client (compliance monitoring). In addition specified scientists designated by the Client may, upon appointment, examine the data.

Group and pen arrangement in animal room

<u>Group:</u> <u>Colour Code</u>	<u>Treatment</u> (mg/kg/day)+	<u>Dog numbers</u>	
		<u>M</u> (even)	<u>F</u> (odd)
1: White	0 (Control)	2- 8	1- 7
2: Yellow	110	10-16	9-15
3: Blue	220	18-24	17-23
4: Red	440	26-32	25-31

	<u>Group/ sex.</u>	<u>Animal no.</u>	<u>Pen no.</u>
Room 6	1F	9	4M 8
	01		28
	2F	10	3M 7
	09		20
	3F	11	2M 6
	17		12
	4F	12	1M 5
	25		04
	1F	13	4M 4
	03		26
	2F	14	3M 3
	11		18
	3F	15	2M 2
	19		10
	4F	16	1M 1
	27		02

	<u>Group/ sex.</u>	<u>Animal no.</u>	<u>Pen no.</u>
Room 7	1F	9	4M 8
	05		32
	2F	10	3M 7
	13		24
	3F	11	2M 6
	21		16
	4F	12	1M 5
	29		08
	1F	13	4M 4
	07		30
	2F	14	3M 3
	15		22
	3F	15	2M 2
	23		14
	4F	16	1M 1
	31		06

+ In terms of Fructose-1,6-diphosphate dehydrated sodium salt (FDP Na3H).

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PROJECTED TIME PLAN

	<u>Date</u>
1. Sample of test compound arrives	<u>18.10.89</u>
2. Animals arrive	<u>03.11.89</u>
3. Treatment commences	<u>28.11.89</u>
4. Terminal sacrifice commences	<u>27.02.90</u>
5. Histopathology completed	<u>#</u>
6. Final report (APC) to Client	<u>#</u>

# To be added

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FDP  
13 WEEK INTRAVENOUS TOXICITY STUDY IN DOGS  
PROTOCOL APPROVAL

For LIFE SCIENCE RESEARCH - ROMA TOXICOLOGY CENTRE

Prepared by: . . . *Marianne Eileraas* . . . . . date: *19-10-89*  
(M. Eileraas)  
(Research Associate, Toxicology)

Checked by: . *Maurizio Franco Mariani* . . . . . date: *19.10.89*  
(M.F. Mariani)  
(Head, General Toxicology)

For IRFI and BIOMEDICA FOSCAMA S.p.A.

This protocol is accepted without revision and my signature authorises the study to proceed as described in this document. This document is the FINAL PROTOCOL for the study, and will be reproduced in the final report.

Approved by: . . . *[Signature]* . . . . . date: *25.10.89*  
(Dr. *R. Scuri*)  
(Study Monitor)

STUDY DIRECTOR

The Client has approved the initiation of this study according to the procedures described in this document. My signature below denotes that I have read and agreed the contents of this document.

. . . . . *Marianne Eileraas* . . . . . date: *31-10-89*  
(M. Eileraas)  
(Study Director)

Person Responsible to  
Italian Ministry of Health : A. Nunziata, Pharm.D., Chem.D.





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PROTOCOL AMENDMENT (1)

Study Title : FDP: 13 Week Intravenous  
Toxicity Study in Dogs  
Study No. : 235-003-009  
Date of issue of Protocol : 19-10-89  
Date of issue of Amendment : Date signed

The following section is to be amended:

1.2 Characteristics

Delete: Stability:

Test substance : 5 years  
Test substance in vehicle : #

Insert: Stability:

Test substance : 5 years  
Test substance in vehicle : At least 1 year

Reason: Missing in original protocol

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PROTOCOL AMENDMENT (1)

FOR LIFE SCIENCE RESEARCH

Amendment prepared by: *Marionne Eileraas* Date: *7-12-89*  
(M. Eileraas - Study Director)

Amendment checked by: *Maurizio F. Mariani* Date: *7.12.89.*  
(M.F. Mariani - Head, General Toxicology)

For IRFI and BIOMEDICA FOSCAMA S.p.A.

Amendment approved by: *[Signature]* Date: *18.12.89*  
(Dott. R. Scuffi - Study Monitor)