



Study code: PCDL-0402

FINAL REPORT

**Acute oral toxicity study of N-acetyl-L-hydroxyproline
with 14-day post-treatment observation period in the rat (limit test)**

Initiation of the study: Feb. 02, 2004
Experimental period: Feb. 12, 2004 to March 02, 2004

Sponsor:
COFOPEX Ltd.
1022 Budapest,
Bimbó út 92.
Contact Person:
István Bara

Study was performed at:
Pharmaceutical Control and
Development Laboratory Co. Ltd.
H-1149 Budapest, Mexikói út 9.
Contact Person:
Susan Somfai-Relle, M.D.

This Final Report consists of 32 pages plus 2 attachments.

2004

**Acute oral toxicity study of N-acetyl-L-hydroxyproline
with 14-day post-treatment observation period in the rat (limit test)
(Study code: PCDL-0402)**

SUMMARY

General information:

Single oral limit dose of 2,000 mg/kg body weight of N-acetyl-L-hydroxyproline (Lot number: 0145003) was applied to rats orally by gavage. Animals were observed for lethality and toxic symptoms for 14 days.

Gross pathological examination was carried out on the 15th day.

Body weight:

The body weight of the animals corresponded to their species and age throughout the study.

Evaluation:

No death occurred after oral administration of N-acetyl-L-hydroxyproline at 2,000 mg/kg dose.

No toxic clinical symptoms were observed.

Scheduled autopsy carried out on day 15 revealed no toxic gross pathological changes.

Conclusion:

No adverse effects were noted at single oral dose of 2,000 mg/kg N-acetyl-L-hydroxyproline in male and female rats.

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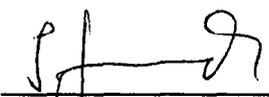
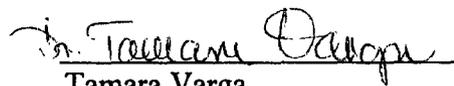
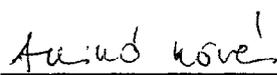
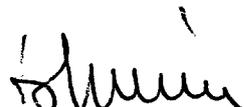
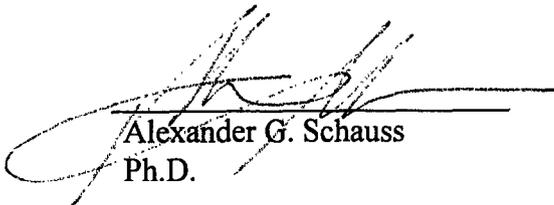
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Copy of Analysis Certificate
Copy of Statement of GLP Compliance (PCDL)

Staff in Charge

	Signature	Date
Director of the Laboratory:	 István Financsek M.D., Ph.D.	<u>22-03-2004</u>
Head of the Toxicological Department, Study Director:	 Susan Somfai-Relle M.D., toxicologist	<u>March 22, 2004</u>
Deputy Study Director:	 Tamara Varga Ph.D., agronomist, toxicologist	<u>March 22, 2004</u>
Quality Assurance Unit:	 Anikó Kövér M. Sc. in Bioengineering	<u>March 22, 2004</u>
Sponsor:	 István Bara Managing Director COFOPEX Ltd.	<u>22-03, 2004</u>
Monitoring Scientist:	 Alexander G. Schauss Ph.D.	<u>April 4, 2004</u>

**Acute oral toxicity study of N-acetyl-L-hydroxyproline
with 14-day post-treatment observation period in the rat (limit test)
(Study code: PCDL-0402)**

Study Director's Statement

I hereby certify that this study report provides a true and complete record of the data generated and that the study was conducted in accordance with the Principles of Good Laboratory Practice as set forth in the following documents:

1. US Food and Drug Administration Title 21, Code of Federal Regulations, Part 58 Good Laboratory Practice Regulations for Nonclinical Laboratory Studies
2. Good Laboratory Practice Regulations (9/2001. EüM-FVM)
3. Hungarian Act 1998: XXVIII. modified by Governmental Regulation 103/2002, regulating animal protection

Date

March 22, 2004

Signature:



Susan Somfai-Relle, M.D.
Study Director

**Acute oral toxicity study of N-acetyl-L-hydroxyproline
with 14-day post-treatment observation period in the rat (limit test)
(Study code: PCDL-0402)**

Statement of the Quality Assurance Unit

This study has been inspected and the report audited by the Quality Assurance Unit of PCDL in compliance with the Principles of Good Laboratory Practice. As far as it can be reasonably established, the methods described and the results incorporated in the report accurately and completely reflect the raw data produced during this study.

Inspections concerning adherence to the protocol were performed:

Date Inspection / Audit	Type of Inspection	Date of Report to the	
		Study Director	Management
Feb. 02, 2004	Protocol audit	Feb. 02, 2004	Feb. 02, 2004
Feb. 17, 2004	Treatment	Feb. 17, 2004	Feb. 18, 2004
March 02, 2004	Autopsy	March 02, 2004	March 03, 2004
March 17, 2004	Draft report audit	March 17, 2004	March 18, 2004

Date: *March 22 2004*

Signature:

Anikó Kövér

Anikó Kövér
M. Sc. in Bioengineering
Head of the Quality
Assurance Unit at PCDL

1. GENERAL INFORMATION

1.1. Title of the study

Acute oral toxicity study of N-acetyl-L-hydroxyproline with 14-day post-treatment observation period in the rat (limit test)

Initiation of the study: Feb. 02, 2004

Experimental period: Feb. 12, 2004 to March 02, 2004

1.2. Objective of the study

To develop data on the potential toxicological effects of single oral administration of N-acetyl-L-hydroxyproline in the rat. The test article has been used in dermatology, to promote wound healing, in rheumatic disorders i.e. osteoarthritis.^{1,2}

1.3. Type of the study

Preclinical toxicological study in compliance with the principles of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies of the United States Food and Drug Administration and the Hungarian Act 1998: XXVIII. modified by Governmental Regulation 103/2002, regulating animal protection. Limit test.

2. TEST AND REFERENCE ARTICLES

2.1. Characteristics of the test article

Name of the article:	N-acetyl-L-hydroxyproline, abbrev: NAHYP
Manufacturer:	KYOWA HAKKO KOGYO CO., LTD. 1-6-1 Ohtemachi, Chiyoda-ku, Tokyo, JAPAN
Lot # :	0145003
Active substance (dry basis):	99.9 %
Specific rotation (at 20°C):	-119°
Foreign amino acids:	not detected (TLC 10 µg)
Loss on drying:	0.7 %
Ammonium (NH ₄):	not more than 0.020 %
Chloride (Cl):	not more than 0.020 %
Sulfate (SO ₄):	not more than 0.028 %
Iron (Fe):	not more than 10 ppm
Heavy metals (Pb):	not more than 10 ppm
Arsenic (As ₂ O ₃):	not more than 1 ppm
Identification number in PCDL:	2004/02068
Certificate of Analysis:	dated : Feb. 20. 2003

Appearance: white crystalline powder
pH (in a solution of 100 mg/ml): 1.78 (measured at PCDL)
Storage conditions: room temperature
Expiration date: 3 years
Analytical examination of all ingredients of the test article had been performed by the Sponsor prior to the study (Certificate of analysis see attached)

2.2. Characteristics of the article used for formulation of the test article

Name: distilled water
Manufactured by: PCDL-TOX
Batch number: A 0040903
Storage conditions: at room temperature
Expiry: 09. 2004

2.3. Characteristics of article used for over-anesthesia before necropsy

Name: T 61
Ingredients: 0.2 g embutramide, 0.005 g tetracaine hydrochloride, and 0.05 g mebezonium iodide per ml
Manufacturer: Intervet International
Batch number: 03 L 004
Storage conditions: at room temperature, in safety box for poisonous drugs
Expiry date: 06. 2008
Dose: 0.1 ml / 100 g body weight

2.4. Formulation of the test article

The necessary amount of the test article was weighed and dissolved in distilled water not earlier than 10 min before administration.

The following solution was prepared:

Nominal dose 2,000 mg/kg: 20,0 g N-acetyl-L-hydroxyproline + distilled water
ad 100 ml

2.4.1. Concentration and homogeneity check of the formulated test article

Concentration and homogeneity of the test article were checked by titrimetry. Homogeneity of the test solution was checked prior to study, 3 samples of 3 ml each were taken from the top, middle and bottom regions. Samples for concentration check were taken from the test solution immediately before the dosing procedure: 3 samples were taken from the middle region. Titrimetry was performed by the Analytical group of PCDL with 0.1 n NaOH solution in the presence of bromothymol blue until green color appeared. 1.00 ml 0.1 n NaOH solution corresponds to 17.317 mg NAHYP.

Results of the homogeneity check

Nominal concentration mg/ml	Actual concentration mg/ml		Difference %	Date of sampling / measurement
200	top	194.58	- 2.71	Feb. 04, 2004
	middle	195.17	- 2.42	
	bottom	194.87	- 2.56	

Results of the concentration check

Nominal concentration mg/ml	Actual concentration mg/ml		Difference %	Date of sampling / measurement
200	1 st sample	197.86	- 1.07	Feb. 17, 2004
	2 nd sample	196.96		
	3 rd sample	198.75		

The actual concentrations were within the acceptable limits of $\pm 5\%$.

2.4.2. Stability control of the test article

Stability control of the test article is the Sponsor's responsibility.

3. TEST SYSTEM**3.1. Animals**

Species / Strain: Sprague Dawley rat, CrI:CD BR

Age at arrival: 6 to 7 weeks (42 to 49 days)

Body weight at arrival: males: 140.4 – 161.3 g

females: 140.0 – 141.8 g

A pool of animals ordered: 30 (15 males, 15 females)

Number of animals involved in the study: 20 (10 males, 10 females)

3.1.1. Origin

Supplier:

Charles River Hungary Ltd.

H-1078 Budapest, István utca 11.

Breeder:

CRD Wiga Ltd.

Sandhofer Weg 7, Sulzfeld, Germany

3.1.2. Hygienic class

SPF at arrival, kept in conventional environment during the study.

3.2. Reason for the selection of species

The rat is commonly used for toxicological studies in accordance with international recommendations. The Sprague Dawley strain is a well-known laboratory model with sufficient historical data.

3.3. Identification and housing of animals

The animals were identified by ear numbering technique and housed in cages by fives of the same sex. The cages were labeled with tags indicating the I.D. numbers of the rats, the study code, the group identification, sex, route of administration, and the starting and ending dates of the experimental period.

3.4. Housing conditions

Hygienic level: conventional
Type of animal cages: type II, macrolone (polycarbonate) bottom
with stainless steel lid
Size of cage: H x W x D : 17.5 cm x 22.5 cm x 37.5 cm
Cleaning: by changing the bedding material containing bottom
of the cages three times a week

Number of animals per cage: 5

Number of animal keeping room: 121

3.4.1. Environmental conditions

Air exchange: approximately 15 times/hour
Temperature: $22 \pm 2^{\circ}\text{C}$
Relative humidity: 40 - 55 %
Lighting: artificial, 12-hour light-dark cycles.

Environmental conditions were maintained by a regulated air-conditioning system, temperature and relative humidity were continuously recorded. (Results are kept in the study file.)

3.4.2. Feed

Free access to standardized rat and mouse diet ssniff SM R/M-Z+H, 15 mm, autoclavable except for the overnight fasting period prior to treatment, during the treatment and for the first two hours of the post-treatment observation.

The composition of the diet and the acceptable level of contaminants were controlled by the Manufacturer ssniff Spezialdiäten GmbH; D-59494 Soest, Germany. The diet is identified by the date of manufacturing (11. 2003), expiry date 03. 2004. Batch number: 206 3246 (List of ingredients is kept in the study file.)

3.4.3. Drinking

Rats had free access to tap water via drinking bottles. Drinking water is checked monthly by the Microbiological Department of PCDL.

3.5. Acclimatization period

The animals were observed for 5 days prior to the treatment. Only healthy animals, free from any clinical symptoms were used in the study.

3.6. Randomization

The animals were assigned to the study on the basis of their body weight so that their individual body weights were in an interval within $\pm 20\%$ of the mean weight of the group at treatment. The within group sequence of the animals was determined with a random table generated by a computer.

4. EXPERIMENTAL DESIGN

4.1. Dose levels, group division

Group number	Treatment	Dose	Number of animals		Identification numbers	
		mg/kg	Males	Females	Males	Females
1	N-acetyl-L-hydroxyproline	2,000	10	-	21 - 30	-
2	N-acetyl-L-hydroxyproline	2,000	-	10	-	31 - 40

4.2. Reason for dose selection

2,000 mg/kg is the highest dose of N-acetyl-L-hydroxyproline which can be given in solution to rats safely and this is the limit dose recommended in the international guidelines for acute toxicity testing. N-acetyl-L-hydroxyproline has been used in doses up to 600 mg daily by mouth.^{1,2} The 2,000 mg/kg limit dose applied in this study, corresponds to about 230 fold the 8.6 mg/kg daily dose if consumed by an adult or 66 fold of the 30 mg/kg daily dose if it is calculated for a child's body weight, i.e. 20 kg.

5. ADMINISTRATION

5.1. Route of administration and reason for the selection

Application was oral by gavage. The route of application was selected in compliance with international guidelines. The oral route is the anticipated route of human exposure to the test article.

5.2. Frequency and duration of application

Single dose.

5.3. Volume of application

The test article was administered in a volume of 10 ml/kg body weight.

5.4. Duration of the experimental period

5 days of acclimatization, treatment's day, 14 days post-treatment observation period including the treatment's day, and the 15th day: autopsy.

6. OBSERVATIONS, EXAMINATIONS**6.1. Lethality**

Observations were made twice daily at the beginning and end of the working day and once on week-end days.

6.2. General state, external appearance, behavior, and clinical symptoms

Careful clinical observation of the rats was carried out once before the exposure, then, after the treatment for 6 hours continuously, and during the subsequent period, animals were checked twice daily for physical signs of toxicity. On week-ends, animals were checked once daily. Signs to be observed included changes in skin, fur, eyes and visible mucous membranes; occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, diarrhea, pupil size, unusual respiratory pattern). Furthermore, potential changes in gait, posture and response to handling as well as the presence of somnolence, trembling, clonic or tonic movements, stereotypes or bizarre behavior were recorded.

6.3. Body weight

Animals were weighed at arrival in the laboratory, on the day of randomization, on the day of treatment before the treatment, as well as on the 2nd, 8th days, and on the 15th day of the experiment prior to autopsy.

7. PATHOLOGY**7.1. Autopsy**

All rats on completion of the post-treatment observation period are exterminated under T61 over-anesthesia and autopsied.

External and internal status were carefully observed and recorded.

No microscopic examination of organs was performed.

8. EVALUATION, STATISTICAL ANALYSIS

Groups of males and females were evaluated separately.

8.1. Parametric values

Individual changes from body weights weighed on Days 1 and 2, Days 2 and 8 as well as Days 8 and 15 were calculated and tabulated (body weight gain). Mean values and standard deviations from the individual body weights and body weight changes were calculated.

8.2. Non parametric values (lethality and clinical symptoms)

The incidence of lethality and clinical symptoms were tabulated.

9. PROCEDURES

The experiments were performed according to the current Standard Operating Procedures of the Department of Toxicology of the Pharmaceutical Control and Development Laboratory Co. Ltd.

10. ANIMAL PROTECTION

In the interests of animal welfare the unnecessary use of animals was avoided. To order the mild extermination of unambiguously moribund animals was the responsibility of the study director. The present method (limit test) uses a reduced number of experimental animals in comparison to other known and acknowledged acute toxicity tests.

11. DATA RECORDING AND ARCHIVATION

All original data were maintained, as dictated by the Standard Operating Procedures, on appropriate forms as follows:

- Test Compound weighing
- Animal room logbook
- Body weight logbooks
- Lethality and Clinical observations logbooks
- Postmortem records

The data obtained in the course of the study were collected in a Study File. The Study Protocol, all data generated during and as a result of the study, the documents and all information in connection with the study, a control sample of the test article, and the Final Report will be stored at least for 15 years in the Archives of the PCDL then offered to the Sponsor.

12. SCHEDULE OF THE STUDY

Arrival of the animals: February 12, 2004
Randomization: February 16, 2004
Treatment day: February 17, 2004
Autopsy: March 02, 2004

13. RESULTS**13.1. Lethality**

(see Table 1. and Appendices 1.1.-1.2.)

No death occurred following the single oral administration of 2,000 mg/kg dose of N-acetyl-L-hydroxyproline to rats. All males and females survived until the end of the 14-day observation period.

13.2. Clinical symptoms

(see Table 2. and Appendices 2.1.-2.2.)

No toxic symptoms were observed on the day of application and during the 14-day post-treatment period at any group of the treated animals.

13.3. Body weights

(see Tables 3.1.-3.2. and Appendices 3.1.-3.4.)

The body weight and the body weight gain of the animals corresponded to their species and age throughout the study.

13.4. Gross pathology

(see Table 4. and Appendices 4.1.-4.2.)

All animals survived until the scheduled autopsy on Day 15 and all proved to be free of toxic pathological changes.

14. EVALUATION

No death occurred after single oral application of 2,000 mg/kg N-acetyl-L-hydroxyproline dose.

No toxic clinical symptoms occurred.

Scheduled autopsy at Day 15 revealed no toxic gross pathological changes.

Conclusion

No adverse effects were noted at single oral limit dose of 2,000 mg/kg N-acetyl-L-hydroxyproline in male and female rats.

Susan Somfai-Relle
Susan Somfai-Relle M.D. *March 27, 2004*
Study Director

References:

¹ Martindale The Extra Pharmacopoeia 29th edition. Ed: James E.F. Reynolds, London The Pharmaceutical Press, 1989, pp 1597

² Martindale The complete drug reference 33rd edition. Ed: Sean C Sweetman, London Chicago Pharmaceutical Press, 2002, pp 1645