REPORT

HEPATOTOXICITY STUDY

OF

UKRAIN®

(AMPOULE 5 MG/5 ML)

IN THE RAT

MARCH 2004

MICHAEL MULLER PhD

CONFIDENTIAL

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Study Statement

This study was conducted at the request of Pharmwiz International for Nowicky Pharma, Margaretenstrasse 7/7, A-1040 Vienna, Austria.

This study was conducted to examine to potential for Ukrain solution for injection (Ampoule 5 mg/5 ml) to induce hepatotoxicity in the rat.

The work for this study was conducted between 22 January 2004 and 15 March 2004 at the laboratories of the ANZAC Research Institute, Concord Repatriation and General Hospital, Concord, NSW 2139, Australia.

On final acceptance of this report by Nowicky Pharma, all original data acquired during the course of the study, in addition to other relevant documents, will be archived at the ANZAC Research Institute for a period of five (5) years. Before or at the end of the five (5) year period Nowicky Pharma, or Pharmwiz International as the agent for Nowicky Pharma, must provide written instructions for the return or disposal of the archived material.

Michael Muller, MA, PhD
16 March 2004

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Tables and Figures

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Summary

Aim of the Study

The aim of this study was to examine to potential for Ukrain solution for injection (Ampoule 5 mg/5 ml) to induce hepatotoxicity in the rat.

Administration

The test substance was administered by intraperitoneal injection to two groups of 5 male and 5 female Sprague-Dawley rats for 5 consecutive days. Injections were made at the same time each day. The doses selected were equivalent to the maximum (0.3 mg/kg/day) and 5 times the maximum (1.5 mg/kg/day) human daily dose.

Investigations

- Body weights and body weight gain
- Observations in life
- Gross liver pathology
- Liver histopathology
- Plasma hepatic enzymes levels

Results

Mortality

All animals survived until the scheduled termination of the study.

Body weights and body weight gain

Body weights and body weight gain were normal.

Observations in life

All animals were normal throughout the study.

Plasma hepatic enzyme levels

There were no increases in plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) or alkaline phosphatase (AP) that were considered treatment-related.

Necropsy

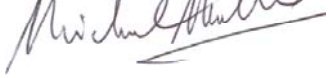
Gross liver pathology was normal. Histopathological examination of the liver revealed no lesions considered to be treatment-related. There were no increases in plasma levels hepatic enzymes that were considered treatment-related.

Sex differences

There were no treatment-related sex differences.

Conclusion

Intraperitoneal injection of Ukrain at 0.3 mg/kg/day or 1.5 mg/kg/day for 5 consecutive days did not induce hepatotoxicity in the rat.



Dr Michael Muller
Study Director

Dr Victoria Cogger
Study Scientist

Good Laboratory Practice Compliance Statement

HEPATOTOXICITY STUDY OF UKRAIN® (AMPOULE 5 MG/5 ML) IN THE RAT

This study was conducted according to the OECD Principles of Good Laboratory Practice, OECD Monograph (as revised in 1997).

Sydney, 17 March 2004

Dr Michael Muller
Study Director

General Information

Sponsor

Nowicky Pharma

Margaretenstrasse 7/7,

A-1040 Vienna, Austria

Test Substance Details

Test substance:	Ukrain® Ampoule (5 mg/5 ml)
Test substance delivery date:	15 October 2003
Batch number of test substance:	310607
Expiry date of test substance:	October 2006

Negative Control Substance Details

Sodium Chloride Injection BP 0.9%

0.9% sterile saline solution (ampoules) 45 mg (0.75 mmol) in 5 ml

Batch number: 17465

Expiry date: June 2004

Supplier: AstraZeneca (Australia)

Responsible personnel:

Study director	Dr Michael Muller
Study scientist	Dr Victoria Cogger
Clinical Chemistry	Dr Margaret Janu
Clinical Pathology	Dr Betty Lin
Animal care	Mamdouh Khalil

Time schedule

Date of protocol	22 September 2003
Date of ethics approval	10 November 2003
First date of dosing	22 January 2004
Last date of dosing	09 February 2004
Experimental completion date (last sacrifice)	10 February 2004
Study completion date	17 February 2004

HEPATOTOXICITY STUDY OF UKRAIN®

(AMPOULE 5 MG/5 ML) IN THE RAT

1. Introduction

The aim of this study was to examine to potential for Ukrain solution for injection (Ampoule 5 mg/5 ml) to induce hepatotoxicity in the rat.

2. Materials and Methods

Ukrain solution (Ampoule 5 mg/5 ml) was provided by Nowicky Pharma in amber glass ampoules. The samples were labelled with the batch number 310607 with an expiry date of October 2006.

On arrival at the test facility the ampoules were examined for seal integrity and then stored in the dark at 22^{o}C prior to use. Each ampoule was found to contain a clear yellow liquid free of particulate matter.

2.1 Animals

2.1.1 Ethics

All experimental procedures used in this study were approved by the Central Sydney Area Health Service Animal Ethics Committee. The animal ethics approval number was 2003/025A.

2.1.2 Source of animals

Specific pathogen free male and female Sprague Dawley rats at 7 weeks of age were purchased from the Animal Research Centre, Perth, Western Australia.

2.1.3 Housing

On arrival at the test facility the animals were examined and place five to a cage where they were allowed to acclimatise for a period of one week. Throughout this time and during the experimental procedures food and water were available to the

animals *ad libitum*. All animals were inspected daily and were housed under a 12 hour light/dark cycle at an average temperature of 22^{o}C.

2.2 Preparation and administration of the test substance

Ukrain solution (1 mg/ml) was made up to the required dose for each animal by dilution with 0.9% saline solution. The pH of the provided Ukrain solution, as stated on the product insert, was between 3.5 to 5.5. The pH was not adjusted prior to use.

Intraperitoneal injections were made between 12:00 and 12:30 pm each day for 5 days. Injections were made using Becton-Dickson Ultra-Fine 29 gauge (0.33 x 12.7 mm) needles.

2.3 Justification for route of administration

Intraperitoneal injections were made in order to deliver a sufficiently large dose to the test animals. The solution provided by the Supplier was 1 mg/ml.

2.4 Procedure

In this study, eight-week-old male Sprague Dawley rats received daily intraperitoneal injections of Ukrain for a period of 5 days. On the sixth day the animals were sacrificed, the livers weighed and prepared for routine histology. Blood samples were taken for analysis of liver enzymes, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AP). Animals were weighed at the start of the study and again immediately prior to necropsy. Animals were observed daily for signs of morbidity.

Fifteen rats of each sex were randomly allocated into three treatment groups: Control, Low Dose Ukrain and High Dose Ukrain (Table 1). Each group contained five animals per sex. Individual animals were identified using an indelible black marker.

Table 1. Treatment Groups

Treatment Group	Animal numbers	Numbers/Sex	Dose (mg/kg/day)	Injection Volume
Control	C1 - C5	5 m	0	0.5
	C6 -C10	5 f		
Low dose	L1- L5	5 m	0.3	0.5
	L6 - L10	5 f		
High dose	H1 - H5	5 m	1.5	0.5
	H6 - H10	5 f		

2.5 Statistical Analysis

Body weights, liver weights and plasma enzyme levels were analysed by one way analysis of variance (ANOVA). Results are presented as the mean ± standard deviation.

3. Results

3.1 Males

The average weights for the male animals are presented in Table 2 and individual male animal weights are presented in Appendix A, Table 8. There were no statistically significant differences in animal weights between groups at the beginning or end of the study. Average animal weights increased slightly across all treatment groups during the study reflecting the expected growth pattern of these rats at this stage of development [1].

Table 2. Average animal weight (males)

Treatment Group	Animal weight (g)* Day 1	Animal weight (g)* Day 6
Control	318 ± 33.1	336 ± 39.2
Low dose	326 ± 38.2	350 ± 53.5
High dose	340 ± 42.6	344 ± 61.7

* values represent the mean ± standard deviation (n=5)

On examination at necropsy no gross abnormalities were observed. There was no evidence of local irritation or peritoneal inflammation at the injection sites.

A comparison of absolute liver weights between treatment groups and control animals showed no statistically significant differences following treatment (Table 3). The individual male absolute liver weights are presented in Appendix A, Table 9.

Table 3. Average absolute liver weight (males)

Treatment Group	Liver weight (g)*
Control	12.8 ± 1.71
Low dose	13.7 ± 2.27
High dose	13.4 ± 2.77

* values represent the mean ± standard deviation (n=5)

On histological examination no treatment-related changes could be detected between livers from the control or treatment groups with no evidence of fibrotic or necrotic changes (Appendix C, Figure 1). One liver from the low dose group had evidence of minor fat deposition (Appendix C, Figure 2). However, the occurrence of this lesion in only one of five animals with no evidence of such lesions at the higher dose indicated a non-treatment related occurrence.

Hepatic Enzymes

No statistically significant changes in plasma levels of ALT, AST or alkaline phosphatase (AP) were observed between control animals and treatment groups (Table 4). Individual animal plasma enzyme levels for males are presented in Appendix A, Table 10.

Table 4. Plasma levels of hepatic enzymes (males)

Treatment Group	ALT (IU/L)*	AST (IU/L)*	AP (IU/L)*
Control	38.6 ± 16.6	68.8 ± 12.8	1.0 ± 0.0
Low dose	41.8 ± 9.5	71.2 ± 15.4	0.8 ± 0.4
High dose	31.0 ± 2.2	58.4 ± 3.0	0.8 ± 0.4

* values represent the mean ± standard deviation (n=5)

3.2 Females

The average weights for the female animals are presented in Table 5 and individual female animal weights are presented in Appendix B, Table 11. There were no statistically significant differences in animal weights between groups at the beginning or end of the study. Average animal weights increased slightly across all treatment groups during the study reflecting the expected growth pattern of these rats at this stage of development [1].

Table 5. Average animal weight (females)

Treatment Group	Animal weight (g)* Day 1	Animal weight (g)* Day 6
Control	201 ± 8.24	216 ± 8.01
Low dose	195 ± 7.41	204 ± 7.42
High dose	210 ± 9.37	212 ± 20.6

* values represent the mean ± standard deviation (n=5)

At necropsy, two low dose females exhibited minor gross hepatic lesions. Following histopathological examination these lesions were not considered treatment-related. There was no evidence of local irritation or peritoneal inflammation at the injection sites.

There were no statistically significant differences in absolute liver weights between the treatment groups and control animals (Table 6). The individual female absolute liver weights are presented in Appendix B, Table 12.

Table 6. Average absolute liver weight (females)

Treatment Group	Liver weight (g)*
Control	7.42 ± 0.97
Low dose	7.94 ± 0.34
High dose	7.3 ± 1.02

* values represent the mean ± standard deviation (n=5)

On histological examination no treatment-related changes could be detected between livers from the control or treatment groups with no evidence of fibrotic or necrotic changes (Appendix D, Figure 3). There was evidence of fat deposition in one animal from the control group (Appendix D, Figure 4).

Hepatic Enzymes

No statistically significant differences in plasma levels of ALT, AST or alkaline phosphatase were observed between control and high-dose treatment animals. A statistically significant difference (p <0.01) was observed between the low-dose female ALT plasma levels compared to the control group (Table 7). As this result represents a 1.7-fold increase in the concurrent control plasma ALT level, and there was no statistically significant difference in the plasma AST level at this dose, nor in the ALT and AST at the high-dose, the result is not considered to be of biological significance. Individual female plasma enzyme levels are presented in Appendix B, Table 13.

Table 7. Plasma levels of hepatic enzymes (females)

Treatment Group	ALT (IU/L)*	AST (IU/L)*	AP (IU/L)*
Control	24.8 ± 6.9	78.0 ± 6.7	0.6 ± 0.5
Low dose	41.6 ± 10.4**	93.8 ± 18.5	0.6 ± 0.5
High dose	32.6 ± 3.0	85.2 ± 10.3	1.0 ± 0.0

* values represent the mean ± standard deviation (n=5); ** p <0.01

4. Conclusions

Treatment by intraperitoneal injection of Ukrain for 5 consecutive days was well tolerated by male and female Sprague-Dawley rats. By this mode of administration, no evidence of hepatotoxicity due to Ukrain at the tested doses was observed. There was no evidence for a response based on sex differences.

5. References

1. Wolfensohn, S. and Lloyd, M. (1994). *Handbook of Laboratory Animal Management and Welfare*. Oxford, Oxford University Press

Appendix A Individual Male Data

Table 8. Individual male animal weights

Group	Animal	Day 1 (g)	Day 6 (g)
Control	C1M	317	353
	C2M	333	391
	C3M	317	285
	C4M	356	332
	C5M	318	336
Low Dose	L1M	271	326
	L2M	376	422
	L3M	326	385
	L4M	316	331
	L5M	326	350
High Dose	H1M	301	399
	H2M	386	256
	H3M	306	407
	H4M	321	333
	H5M	340	344

Table 9. Individual male absolute liver weights

Group	Animal	Liver weight (g)
Control	C1M	11.6
	C2M	13.8
	C3M	15.2
	C4M	10.9
	C5M	12.7
Low Dose	L1M	11.4
	L2M	12.6
	L3M	15.7
	L4M	16.5
	L5M	12.2
High Dose	H1M	11.5
	H2M	16.1
	H3M	11.6
	H4M	16.7
	H5M	11.0

Table 10. Individual male plasma enzyme levels

Group	Animal	ALT (IU/L)	AST (IU/L)	AP (IU/L)
Control	C1M	67	88	1
	C2M	38	72	1
	C3M	33	64	1
	C4M	30	67	1
	C5M	25	53	1
Low Dose	L1M	53	79	1
	L2M	36	55	1
	L3M	51	85	0
	L4M	37	83	1
	L5M	32	54	1
High Dose	H1M	32	56	1
	H2M	30	56	0
	H3M	31	60	1
	H4M	28	63	1
	H5M	34	57	1

Appendix B Individual Female Data

Table 11. Individual female animal weights

Group	Animal	Day 1 (g)	Day 6 (g)
Control	C1F	197	241
	C2F	215	196
	C3F	211	192
	C4F	204	208
	C5F	221	193
Low Dose	L1F	186	207
	L2F	200	211
	L3F	206	218
	L4F	197	225
	L5F	188	209
High Dose	H1F	204	202
	H2F	198	200
	H3F	194	195
	H4F	195	216
	H5F	214	199

Table 12. Individual female absolute liver weights

Group	Animal	Liver weight (g)
Control	C1F	8.0
	C2F	6.8
	C3F	6.4
	C4F	8.8
	C5F	7.1
Low Dose	L1F	7.7
	L2F	7.8
	L3F	7.6
	L4F	8.4
	L5F	8.2
High Dose	H1F	8.6
	H2F	8.2
	H3F	6.6
	H4F	6.8
	H5F	6.3

Table 13. Individual female plasma enzyme levels

Group	Animal	ALT (IU/L)	AST (IU/L)	AP (IU/L)
Control	C1F	33	85	1
	C2F	26	81	1
	C3F	27	78	0
	C4F	24	79	0
	C5F	14	67	1
Low Dose	L1F	48	83	1
	L2F	28	70	1
	L3F	37	119	0
	L4F	55	101	1
	L5F	40	96	0
High Dose	H1F	30	78	1
	H2F	29	90	1
	H3F	34	81	1
	H4F	36	101	1
	H5F	34	76	1

Appendix C Male Liver Histology

Figure 1. Male Liver Histology

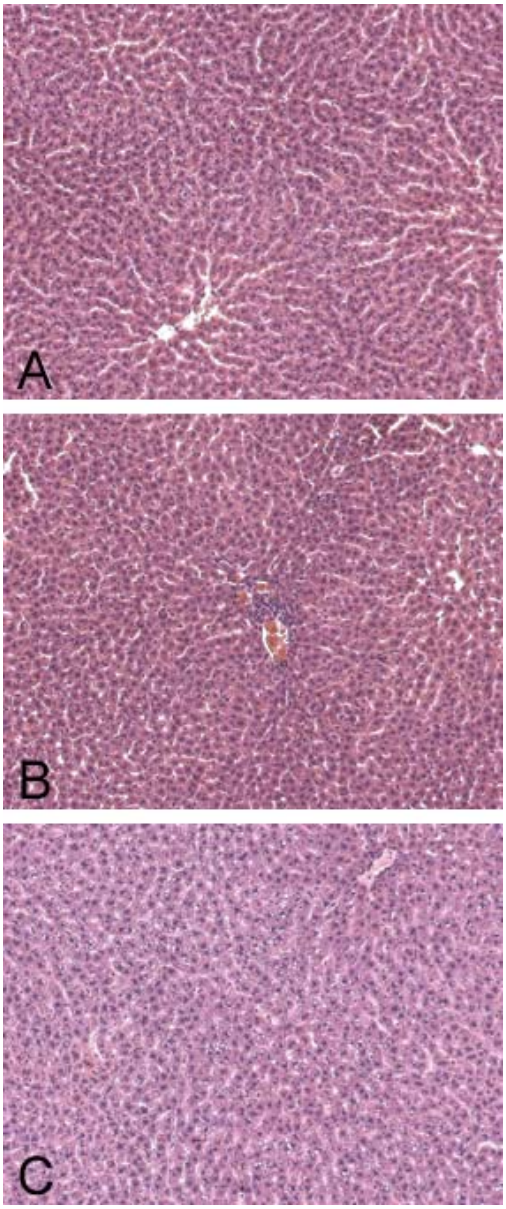


Figure 1: Haematoxylin and Eosin stains of livers from 5-day study of Ukrain hepatotoxicity in male rats. A. Control, B. Low Dose, C. High Dose. Original magnification 400×

Figure 2. Lipid Deposits – Males

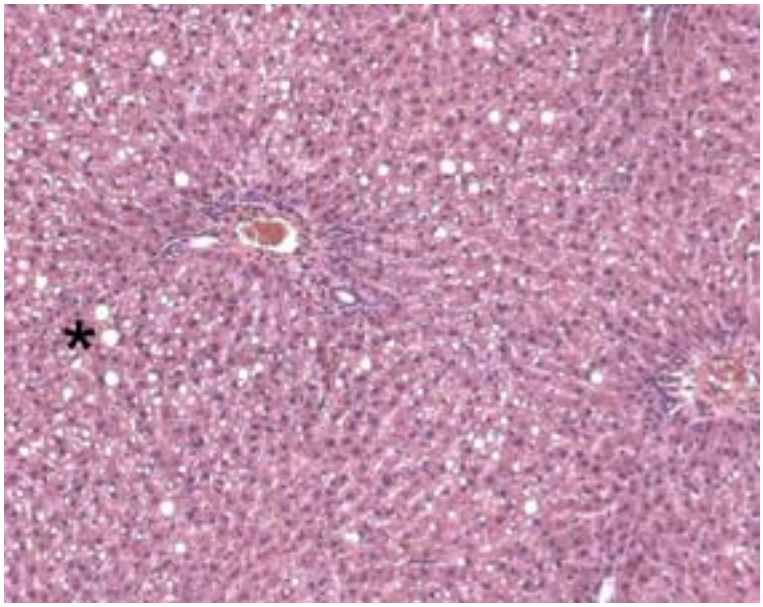


Figure 2: Haematoxylin and Eosin stains of liver from Low Dose group of the 5-day study of Ukrain hepatotoxicity in male rats. Note vacuoles (*), representative of lipid deposition. This change was seen in one rat only.

Appendix D Female Liver Histology

Figure 3. Female Liver Histology

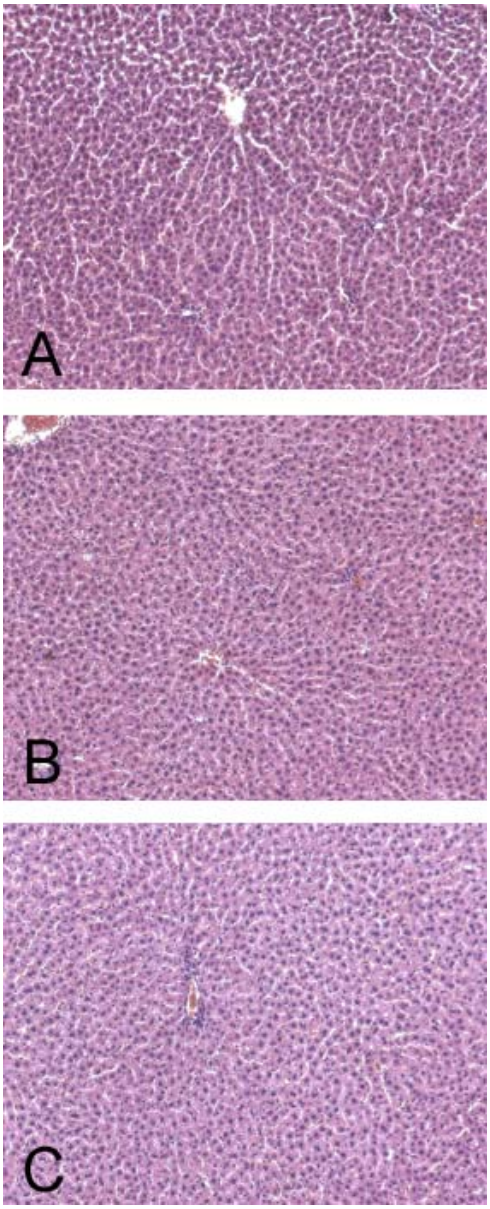


Figure 3: Haematoxylin and Eosin stains of livers from 5 day study of Ukrain hepatotoxicity in female rats. A. Control, B. Low Dose, C. High Dose. Original magnification 400×

Figure 4. Lipid Deposits- Females

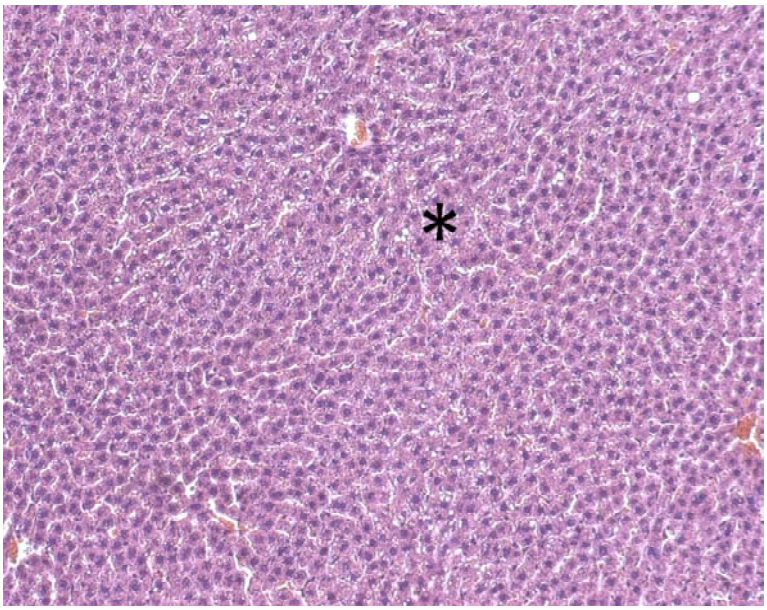


Figure 4: Haematoxylin and Eosin stains of liver from the control group of the 5-day study of Ukrain hepatotoxicity in female rats. Note vacuoles (*), representative of lipid deposition. This change was seen in one rat only.