Oral Acute Toxicity of Hepalip Forte¨ in Rats

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Abstract

The main active component of preparation HEPALIP FORTE® is EPL - essential phospholipids. Their chemical structure corresponds to that of endogen phospholipids, but they have functional superiority because of the content of unsaturated fatty acids. Essential phospholipids in combination with the vitamins have been used in the treatment of liver diseases, dyslipoproteinaemias and intoxications with consequent liver failure. Acute toxicity study on **HEPALIP FORTE®** was performed on Wistar rats. The main aim of toxicology studies for the drug registration process is evaluation of the toxic potential and risks of human exposition to the substance (Gelbke et al., 1999). Acute toxicity is an orientation point of the test substance toxicity and represents a starting test for the toxicological evaluation.

Study included one oral dose of the substance, applied with oesophageal intubations. There were three dose-levels: 300, 500 and 1000 mg/kg. No lethality was recorded and statistical analysis of body weight variations failed to show any significant difference between the groups. Reversible tremor was more frequently recorded in females and was not present in control animals. After the planed sacrifice, no changes related to the test substance were recorded. We noticed a statistically significant difference in the liver weights between males of 3M and 2M groups in comparison to the control. Similar (not significant) tendency was noticed in females. Significant differences in organ weights might be suggestive of a toxic effect that experimental animal managed to recover from in partial manner. The histopathological analysis detected no changes in the structure and morphology of liver parenchyma.

Key words: acute toxicity, essential phospholipids.

Introduction

Essential phospholipids (EPL) are present in many biological systems, especially in lipoprotein complexes, in membrane structures or in lipid particles in circulation. Essential phospholipids facilitate the regulation of important biochemical processes in different biological systems, like cardiovascular and nervous system.

Their chemical structure corresponds to that of endogen phospholipids, but they have functional superiority because of their content of unsaturated fatty acids. Essential phospholipids incorporate in the membrane and organelle systems of cells, and become the constitutive elements of cellular membranes. Membrane metabolic processes are responsible for the whole cell regeneration. The membrane enzymes are more active in the presence of phospholipids, and unsaturated fatty acids are precursor parts of the future prostaglandin molecule (arachidonic acid). By stabilizing the membrane, phospholipids prevents the peroxidation of lipids, formation of the free radicals and up-regulation of the LDL receptors (Nieman et al., 1990, Skakun and Stepanova, 1986).

Essential phospholipids in the combination with vitamins have been used in the treatment of liver diseases, dyslipoproteinaemias and intoxications with liver failure. In animal toxicology studies of essential phospholipids, the toxicity was minimal (documentation of the Institute of Pharmacology and Toxicology, Sarajevo). The aim of this acute toxicity study is to investigate and confirm all previously mentioned results with the usage of **HEPALIP FORTE®.**

Material and methods

The study was performed on the albino Wistar rats. During the randomisation, animals with unsatisfying body weight or health condition were excluded. Animals were randomised in three experimental and one control group, each of 5 males and 5 females. Animals were in quarantine for 2 weeks. During that time their health condition was monitored and eventual symptoms were recorded.

Study included one oral dose of the test substance, applied by oesophageal intubations. Test substance application day was the first day of the observation period that lasted 14 days. There were three dose-levels: 300, 500 and 1000 mg/kg. Control animals were treated with the vehicle (milk). Dose concentrations of the test substance were prepared just before the application.

The test samples were **HEPALIP FORTE®** capsules, manufactured by Bosnalijek d.d. Sarajevo. The content of capsules was dissolved in milk heated in the water bath up to 60°C. Composition of the each capsule is:

- Active components: EPL substance 300 mg, Nicotinamide 30 mg, Pyridoxine hydrochloride 6 mg, Riboflavin 6 mg, Thiamine nitrate 6 mg, Tocopherol acetate 6 mg;
- Other constitutes: *triglycerida saturata media*, hard gelatinous capsule N°1.

Four clinical examinations were performed during the first day after the test substance application, in 2-hour intervals. Clinical examinations and mortality check-ups were carried out every day in the invariable time intervals, in the morning and evening. During the whole study, the environmental conditions were kept constant, with daily recording of the temperature and humidity. Determination of the body weights was done before the test substance application, after 7 days and at the end of the study. Intended each animal sacrifice with necropsy was performed after the observation period. Macroscopic examination and determination of the organ weights were carried out in all animals.

Results

There was no lethality during the study. The following parameters were recorded:

- Periodical body weight measurements
- Respiration rate
- Eventual toxic signs
- Determination of the organ weights after sacrifice

Statistical analysis was done in the Microsoft® Excel 2002 and SigmaStat for Windows 2.03 programs. Experimental animals were randomised in 8 groups (4 male and 4 female groups). Groups were similar according to their characteristics and consisted of five animals each. In the text that follows, all animal groups will be coded because of the easier result presentation. Animals in the groups 1, 2 and 3 were treated with following single doses: "1" - 300 mg/kg, "2" - 500 mg/kg and "3" - 1000 mg/kg. "K" letter is a sign for the control animals.

Experimental animals were randomised, so the mean body weight did not significantly differ between groups (p=0.5). Variations inside experimental groups were within two standard deviations. Animal body weights before the test substance application and before the planned sacrifice and necropsy are presented in Tables 1 and 2.

Table 1. Body weight before application of test substance

Number	Group	Number Of animals	Group mean body weight in gram	Standard deviation	Group total body weight in grams
1	3M	5	434.2	35.25	2171
2	2M	5	436	27.91	2180
3	1M	5	443.8	12.48	2219
4	KM	5	450.2	15.02	2251
5	3F	5	281.2	15.71	1406
6	2F	5	301	29.64	1505
7	1F	5	320.2	27.88	1601
8	KF	5	296.2	37.56	1481

Table 2. Animal body weights before sacrifice

Number	Group	Number of animals	Group mean weight mean in grams	Standard deviation	Group total body weight in grams	Difference between total body weights in grams
1	3M	5	422.6	25.32	2113	-42
2	2M	5	415.6	30.14	2078	-102
3	1M	5	430.4	15.5	2152	-67
4	KM	5	429.8	14.86	2149	-2
5	3F	5	278.8	17.12	1394	-12
6	2F	5	293.6	22.1	1468	-37
7	1F	5	314.8	23.42	1574	-27
8	KF	5	287.4	36.14	1437	-44

Table 3. Differences in mean body weights between male groups

Difference between the groups	N	Mean	Std. Dev.	SEM
3M	5	11.600	12.442	5.564
KM	5	20.400	8.355	3.736
Difference: -8.800 t = -1.313 with 8 degrees of freedom (P = 0.226)				
Difference between the groups	**N**	**Mean**	**Std. Dev.**	**SEM**
2M	5	20.400	6.914	3.092
KM	5	20.400	8.355	3.736
Difference: 0.000 t = 0.000 with 8 degrees of freedom (P = 1.000)				
Difference between the groups	**N**	**Mean**	**Std. Dev.**	**SEM**
1M	5	13.400	5.639	2.522
KM	5	20.400	8.355	3.736
Difference: -7.000 t = -1.553 with 8 degrees of freedom (P = 0.159)				

Table 4. Differences in mean body weights between female groups

Difference between the groups	N	Mean	Std. Dev.	SEM
3F	5	2.400	6.427	2.874
KF	5	8.800	3.271	1.463
Difference: -6.400 t = -1.985 with 8 degrees of freedom (P = 0.082)				
Difference between the groups	**N**	**Mean**	**Std. Dev.**	**SEM**
2F	5	7.400	7.668	3.429
KF	5	8.800	3.271	1.463
Difference: -1.400 t = -0.376 with 8 degrees of freedom. (P = 0.717)				
Difference between the groups	**N**	**Mean**	**Std Dev**	**SEM**
1F	5	5.400	10.922	4.885
KF	5	8.800	3.271	1.463
Difference: -3.400 t = -0.667 with 8 degrees of freedom. (P = 0.524)				

Statistical analysis of the body-weight variations was performed separately for males and females. T-test for the independent specimen failed to show any significant difference between the groups. These results are presented in Tables 3 and 4.

Respiratory rates recorded in experimental groups and in control group were within physiological limits for rats. Results of the recorded times are presented in Table 5 while the statistical analysis with descriptive statistics is demonstrated in Table 6.

Tremor was recorded more frequently in females. It was more intensive in females, as well. Tremor as a clinical

Table 5. Respiratory rate on the day of application

Respiratory rate 21.02.2003. 19:30h									
		Group 3M	Group 2M	Group 1M	Control M	Group 3F	Group 2F	Group 1F	Control F
	Animal1	88	76	84	64	68	72	100	60
	Animal2	104	60	108	88	60	60	92	120
	Animal3	84	84	68	68	120	92	96	60
	Animal4	80	68	80	72	68	60	72	60
	Animal5	112	84	60	92	80	88	60	68
	Mean	93,6	74,4	80	76,8	79,2	74,4	84	73,6
	St. Dev.	13.74045	10.43072	18.3303	12.45793	23.89979	15.12614	17.20465	26.16868
	Max	112	84	108	92	120	92	100	120
	Min	80	60	60	64	60	60	60	60

Table 6. Descriptive statistics for respiratory rate

Group	Number of recordings	Mean	Std. Dev.	Std. Error	C.I. of Mean
3M	5	93.600	13.740	6.145	17.061
2M	5	74.400	10.431	4.665	12.951
1M	5	80.000	18.330	8.198	22.760
KM	5	76.800	12.458	5.571	15.469
3F	5	79.200	23.900	10.688	29.676
2F	5	74.400	15.126	6.765	18.782
1F	5	84.000	17.205	7.694	21.362
KF	5	73.600	26.169	11.703	32.493

sign was registered in four females from the group treated with the highest test substance dose, in three females from the group treated with intermediate test substance dose and in three females from the group treated with lowest test substance dose. That sign was not observed in control animals. Only mild tremor was noticed in one male from the group treated with the highest test substance dose.

Statistically, tremor was registered in 18 records - 11.25% (from 160 records in total); 8 times in the 3F group - 4 animals, 6 times in the 2F group - 3 animals and 4 times in the 1F group - 2 animals. Tremor was found to be mild in 13 cases (72.22%) and moderate in 5 cases (27.78%). In almost all cases (94.5%) tremor was registered during the first day of the test substance application (8-10 hours after the test substance application).

Sporadically the hoarse animal phonation was registered, especially in females. That sign was also registered in the control group of animals. It was most frequently expressed during the 3rd and 4th day of the observation period. Such distribution of the tremor expression indicates that it was not being a consequence of the test substance treatment.

A loose faeces was observed sporadically, more frequently in male animals. That sign was also present in control animals and its distribution was similar throughout all days of the observation period. Such distribution of the loose faeces expression indicates that it was not being a consequence of the test substance treatment. Loose faeces were recorded 13 times in males (3.61% of 360 records in total), 4 times in the 3M group, 2 times in the 2M group, 3 times in the 1M group and 4 times in the control group of animals. Loose faeces were found to be mild in 6 cases and moderate in 7 cases. Loose faeces were recorded 4 times in females (1.11%), 1 case in the each group of animals. Loose faeces were found to be mild in 1 case and moderate in 3 cases.

In all animals, macroscopic examination and necropsy were performed after the planned sacrifice. No changes related to the test substance application were recorded. A mild heart hypertrophy was observed in one male from the control group and one male from the intermediate dose group. One male from the control group had the right kidney haematoma. Ovarian hyperaemia was found in 2 females from the high-dose group, 2 females from the intermediate-dose group, 1 female from the low-dose group and 1 female from the control group. Encapsulated

Table 7. Liver weight after sacrifice

	3M	2M	1M	KM	3F	2F	1F	KF
Animal1	12.28	12.27	13.47	15.57	9.52	8.59	11.34	11.68
Animal2	12.77	13.3	13.92	15.03	9.55	10.8	8.86	11.59
Animal3	12.28	10.99	13.7	14.49	8.25	8.94	13.41	7.76
Animal4	12.63	13.56	11.29	14.14	8.44	9	10.06	10.26
Animal5	10.38	12.88	15.22	12.5	8.24	9.28	8.84	10.9
TOTAL	**60.34**	**63**	**67.6**	**71.73**	**44**	**46.61**	**52.51**	**52.19**
Mean	**12.068**	**12.6**	13.52	**14.346**	8.8	9.322	10.502	10.438
St. Dev.	0.967972	1.023597	1.4193132	1.16586	0.675759	0.861928	1.924349	1.603627
Median	12.28	12.88	13.7	14.49	8.44	9	10.06	10.9
Max.	12.77	13.56	15.22	15.57	9.55	10.8	13.41	11.68
Min.	10.38	10.99	11.29	12.5	8.24	8.59	8.84	7.76
Differences	2.278	1.746	0.826		1.638	1.116	-0.064	

tumour formation on the right lung lobus superior was found in 1 female from the control group. The solid, round tumour mass on the lungs was found in another female animal from the intermediate-dose group.

Organ weights of hearth, liver, kidneys, lungs, brain, spleen and thyroid gland were determined in females. Testis weight was determined in males, as well. A statistically significant difference in the liver weights was noticed in the male groups 3M (t= -3.362) with 8 degrees of freedom (P=0.010) and 2M (t= -2.516) (P=0.036) in comparison to the control group of animals. Significant differences in female organ weights were not noticed. Liver weights of experimental animals are presented in Table 7.

Percentile ratio between the organ weights and the total body weight of all groups was analysed. In the 3M group for instance, number 2.779 for the liver means that the weight of all livers in comparison with the total body weight of the 3M group was 2.779%. If we take a look by the groups, an increase and a difference between the 3M and the control group can be noticed (14.68%). Graphical presentation of the percentile ratio of organ weights by the groups is in Graphs 1 and 2.

Discussion

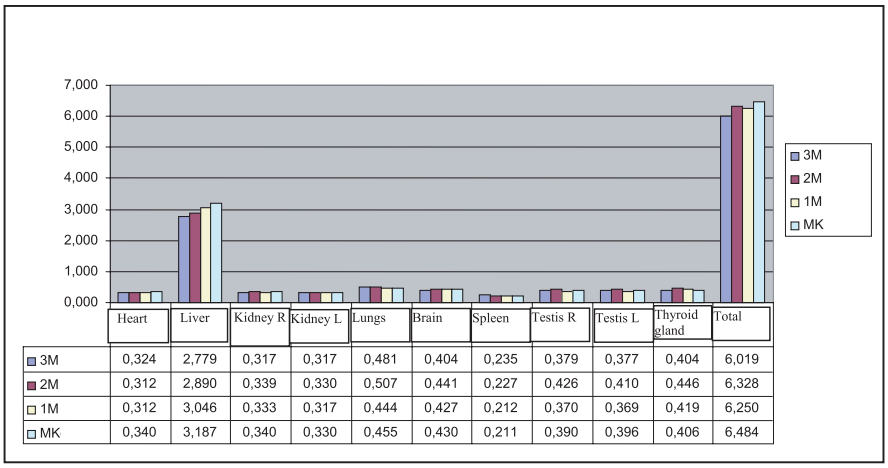
The main aim of the toxicology studies for the drug registration process is evaluation of the toxic potential and risks of human exposition to the substance (Gelbke et al., 1999). The target organ toxicity, dose dependence, exposition to the test substance and potential reversibility of the toxic signs need to be determined. Gathered information is necessary for the determination of the initial dosing in the clinical studies and identification of the parameters for the clinical monitoring and detection of eventual adverse effects (ICH, 1997).
Acute toxicity is giving the orientation about the toxicity of the test substance and is the starting test for the toxicological evaluation. The Organization for Economic Cooperation and Development (OECD) is defining acute toxicity as the toxic effects recorded soon after the application of single oral dose of the test substance or multiple doses applied within 24 hours. By definition, that is statistically calculated dose that is expected to kill 50% of the experimental animals (Chan & Hayes, 1989).

During this study there was no lethality and we can conclude that the test substance in rationally high doses demonstrated low level of toxicity. A substance with LD50 between 0.5 and 5 g/kg is classified as moderately toxic (Lu, 1996). We did not register any lethality after the 1 g/kg-dose application, so the test substance may be classified as substance with moderate toxicity, low toxicity or practically with no toxicity.

When the chemical substance produce toxicity only in certain circumstances, e.g. in extremely high doses, it is not necessary to evaluate the effects of irrationally high doses to demonstrate the toxicity. Information that substance in rationally high doses does not induce any lethality can be sufficient. Dose of 2 g/kg is accepted as a limit dose by the FAO/WHO Expert Committee on Food Additives (WHO, 1996), while US Environmental Protection Agency (EPA, 1994) accepts the limit of 5 g/kg.

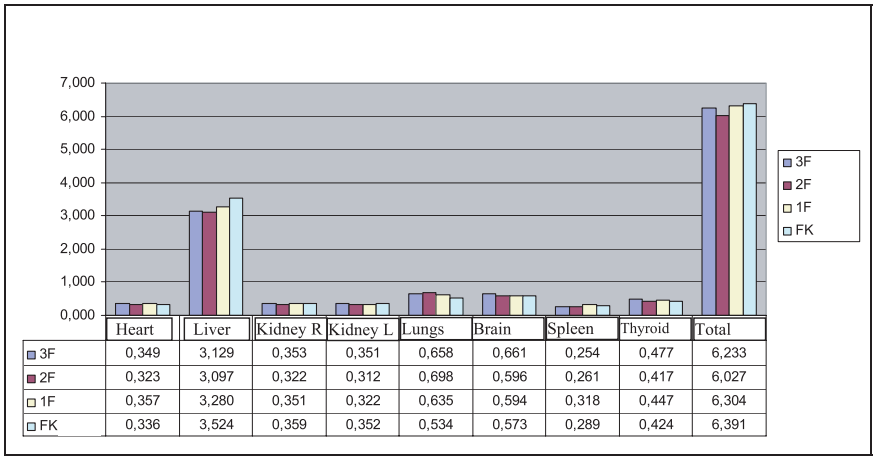
Applied doses in our study are approximations of the proposed future human single (therapeutic) doses. If one capsule (399 mg) contains therapeutic human dose, the applied doses in our study correspond to following:
- A dose of 300 mg/kg corresponds to approximately 50 times higher dose (52.6 times higher)
- A dose 500 mg/kg corresponds to approximately 85 times higher dose (87.7 times higher)
- A dose 1000 mg/kg corresponds to approximately 175 times higher dose (175.4 times higher).

Graph 1. Percentile ratios between organ weights in males



	Heart	Liver	Kidney R	Kidney L	Lungs	Brain	Spleen	Testis R	Testis L	Thyroid gland	Total
3M	0,324	2,779	0,317	0,317	0,481	0,404	0,235	0,379	0,377	0,404	6,019
2M	0,312	2,890	0,339	0,330	0,507	0,441	0,227	0,426	0,410	0,446	6,328
1M	0,312	3,046	0,333	0,317	0,444	0,427	0,212	0,370	0,369	0,419	6,250
MK	0,340	3,187	0,340	0,330	0,455	0,430	0,211	0,390	0,396	0,406	6,484

Graph 2. Percentile ratios between organ weights in females



	Heart	Liver	Kidney R	Kidney L	Lungs	Brain	Spleen	Thyroid	Total
3F	0,349	3,129	0,353	0,351	0,658	0,661	0,254	0,477	6,233
2F	0,323	3,097	0,322	0,312	0,698	0,596	0,261	0,417	6,027
1F	0,357	3,280	0,351	0,322	0,635	0,594	0,318	0,447	6,304
FK	0,336	3,524	0,359	0,352	0,534	0,573	0,289	0,424	6,391

We believe that the testing of the higher test substance doses is not rational, considering the fact that the overdose with a dose 200 times higher than the single therapeutic dose is hardly possible. Some authors consider that testing of the doses 100-300 times higher than the proposed human doses is enough (Gad & Changelis, 1998).

Clinical signs can be reversible and irreversible. The reversible signs are subsiding as the substance is eliminated from the body and generally are not accompanied with permanent damages (Chan et al., 1982). Tremor found in our study was reversible. Maximally tolerated dose considering the tremor records was different for males and females. For female animals, maximally tolerated dose was below the 300 mg/kg, while for male animals that was a dose of 500 mg/kg.

Variation in body weights might be considered as sensitive, but non-specific indicator of the animal general health condition (Gad & Changelis, 1998), especially in subchronic and chronic toxicity studies. The comparison of body weights, at the beginning and at the end of the study, suggests the mild decrease in body weight. That decrease was not found to be greater than 10% of the mean body weight of animals. It was also detected in the control animal groups, so weight decrease could not be an absolute consequence of the substance application. Statistically significant differences in mean body weights between animal groups were not detected.

Macroscopic changes in the acute toxicity studies rarely indicate the toxicity that is going to be seen after the chronic substance administration. The weight determination of organs in chronic studies is common, but some authors suggest that this can be of little value in acute toxicity studies (Gad & Changelis, 1998). Significant differences in organ weights can be suggestive of the toxic effects that experimental animal manage to partially recover from. Differences in ratio between organ and body weights might also be suggestive of the presence of histopathological changes in target organs, especially in

animals sacrificed 1-4 days after the dose application.

We noticed a statistically significant difference in the liver weights between males of the 3M and 2M groups in comparison with the control group of animals. Similar tendency, but statistically not significant was noticed in females. These findings suggested eventual changes in the liver parenchyma and a need for the histopathological analysis. Histopathological analysis was performed and no changes were detected in the structure and morphology of the liver parenchyma.

Conclusion

After statistical analysis of data gathered throughout acute toxicity testing we can conclude the following:

1. Body weight: the test substance application did not induce statistically significant differences in body weights between experimental and control animals.
2. Respiratory rate: the test substance application did not induce statistically significant differences in respiratory rates between experimental and control animals.
3. Recorded tremor: the test substance application did not induce statistically significant differences in recorded tremor between experimental and control animals - free assumption of the investigator.
4. Recorded loose faeces: the test substance application did not induce statistically significant differences in recorded faeces quality between experimental and control animals - free assumption of the investigator.
5. Recorded abnormal phonation: the test substance application did not induce statistically significant differences in recorded phonation quality between experimental and control animals - free assumption of the investigator.
6. Organ weights after necropsy: the test substance application induced statistically significant differences in liver weights between experimental (groups 3M and 2M) and control animals.

Considering these findings, we can conclude that test substance did not express acute toxicity when applied orally in rats, in single doses of 300 mg/kg, 500 mg/kg and 1000 mg/kg.

References

1. Chan P, O Hara G and Hayes A (1982): Principles and methods for acute and subacute toxicity. In: Principles and Methods of Toxicology. Ed. A Hayes. Raven Press NY: 1-51.
2. Chan PK, Hayes AW (1989): Principles and Methods for Acute Toxicity and Eye Irritancy. In: Principles and Methods of Toxicology, Second Edition. Ed. Wallace Hayes. Raven Press, Ltd., NY.
3. Gad CS & Chengelis PC (1998): Acute Toxicology Testing. Second edition. Academic Press, USA.
4. Gelbeke H-P, Hildebrand B & Kerfoot JE (1999): Evaluation of Methods for the Registration of Chemicals. In: Toxicology. Eds. Marquardt H, Schafer GS, McClellan R & Welsch F. Academic Press.
5. ICH (1997): Guidance for Industry M3 Non-clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals.
6. Lu C. Frank (1996): Basic Toxicology Fundamentals, Target Organs and Risk Assessment, Third edition. Taylor & Francis, Philadelphia, USA.
7. Nieman DC, Haig JL, Fairchild KS, De Guia ED, Dizon GP, Register UD (1990): Reducing-diet and exercise-training effects on serum lipids and lipoproteins in mildly obese women. Am J Clin Nutr 1990 Oct; 52(4): 640-5
8. OECD (1981): OECD guidelines for testing chemicals. OECD Paris.
9. Skakun NP, Blikhar EI (1986): Effect of Antioxidants on Lipid Peroxidation and Liver Condition in Patients with Pulmonary Tuberculosis. Farmakol. Toxikol., 1986; 49 (5): 112-114.
10. US Environmental Protection Agency (1994): Health Effects Test Guidelines. Hazard Evaluation. Code of Federal Regulations, Title 40, Part 792 and 798.
11. WHO (1966): Specifications for Identity and Purity and Toxicological Evaluation of Food Colours. WHO. Food Ads./66.25. Geneva: WHO.