2010 - Citotoxicity examination of Kaqun water in HepG2 cells /NICS/

NATIONAL INSTITUTE OF CHEMICAL SAFETY

DEPARTMENT OF RESEARCH FOR CHEMICAL SAFETY

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Closing Report

Number of examination: 02-CTOX-10

Citotoxicity examination of Kaqun water in HepG2 cells

2010

Budapest

Citotoxicity examination of Kaqun water in HepG2 cells

Responsible Persons

	Signature	Date
Principal investigator	(illegible signature) Dr. Zsuzsanna Kocsis Biologist	20.12.2010
Department Head of OKBI-KBKF-MSBO	(illegible signature) Dr. Zoltán Macsek Ph.D. Biologist	20.12.2010
Department Head of OKBI-KBKF	(illegible signature) Dr. Jenő Major Ph.D. Biologist	20.12.2010
Director General of OKBI	(illegible signature) Dr. Imre Bordás Ph.D. Chief Physician	20.12.2010
Head of Quality Control Group of OKBI-KBKF	(illegible signature) Dr. Márta Kovács Pharmacist	20.12.2010

1. PRINCIPAL INVESTIGATOR'S DECLARATION

I the undersigned hereby declare that the toxicity examination titled Citotoxicity examination of Kaqun water in HepG2 cells (with examination number: 02-CTOX-10) was carried out in compliance with the regulations of OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98)17) at the Molecular and Cell Biological Department of the Department of Research for Chemical Safety of the National Institute of Chemical Safety (OKBI).

The examination was carried out based on the decrees titled Biological evaluation of medical devices Part 5: Tests for citotoxicity: *in vitro* methods (ISO 10993-5: 1992), MSZ EN 30993-5:1998; Biological evaluation of medical devices Part 12: Sample preparation and reference materials (ISO 10993-12: 2007), MSZ EN 10993-12:2008.

The examination was carried out according to Standard Operations Regulations of OKBI-KBKF-MSBO.

The Closing Report is based on correct examination data and the obtained results are in compliance with the content of the Closing Report.

Budapest, 20/12/2010

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Dr. Zsuzsanna Kocsis

Principal Investigator

QUALITY ASSURANCE DECLARATION

Title of examination: Citotoxicity examination of Kaqun water in HepG2 cells

Number of examination: 02-CTOX-10

The examination took place observing the (ENV/MC/CHEM(98)17) Guidelines of OECD and no. 9/2001(III:30)EüM-FVM Joint Decree of the Ministry of Health and Ministry of Agriculture and Rural Development on "implementing and checking good laboratory practice".

The examination and Closing Report were audited by the Quality Control Group of OKBI-KBKF. The data published in the Closing Report as well as the methods and procedures applied in the examination reflect the raw data.

Dates of checking	Examination phases	Report dates	
		Principal Investigator	GLP management
04/11/2010	Draft examination plan 1	04/11/2010	04/11/2010
04/11/2010	Final examination plan	04/11/2010	-
10/11/2010	Treatment	10/11/2010	-
12/11/2010	Measuring optical density	12/11/2010	-
17-20/12/2010	Draft Closing Report 1	20/12/2010	20/12/2010
12/10/2010	Closing Report	20/12/2010	-

Budapest, 20/12/2010

(illegible signature)

Dr. Márta Kovács

Head of Quality Control Group

2. SUMMARY

Title of examination:	Citotoxicity examination of Kaqun water in HepG2 cells
Examination material:	Kaqun water for bathing
Examination concentrations:	Without dilution
Examined parameter:	Citotoxicity
Method:	MTT assay
Exposition time:	24 hours
Result	negative

Result of the citotoxicity examination

	Measurement results		Paint-reduction %	Evaluation
	Average	Standard deviation		
Positive control	0.047	0.005	27.16	Positive
DMEM with Kaqun water	0.189	0.024	109.2	Negative
DMEM with ultra-pure water	0.185	0.021	106.9	Negative
DMEM	0.173	0.031	100	Negative

3. Summary

In the given experimental conditions Kaqun water did not reduce the number of viable cells compared to the untreated control group.

Kaqun water does not have any citotoxic effect.

4. GENERAL INFORMATION

4.1. Title of examination

Citotoxicity examination of Kaqun water in HepG2 cells

4.2. Aim of examination

The aim of the examination is to assess the citotoxicity causing effect of Kaqun water.

4.3. Method of examination

The citotoxicity examination was carried out in compliance with the standards titled Biological evaluation of medical devices Part 5: Tests for citotoxicity: *in vitro* methods (ISO 10993-5: 1992), MSZ EN 30993-5:1998; Biological evaluation of medical devices Part 12: Sample preparation and reference materials (ISO 10993-12: 2007), MSZ EN 10993-12:2008.

The examination took place observing the regulations of no. 9/2001(III:30)EüM-FVM Joint Decree of the Ministry of Health and Ministry of Agriculture and Rural Development on "implementing and checking good laboratory practice", of OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98)17), and of OECD The Application of the Principles of GLP to the in vitro Studies (ENV/JM/Mono(2004)26).

4.4. Place of examination

National Institute of Chemical Safety

Department of Research for Chemical Safety Molecular and Cell biological Department

1096 Budapest, Gyáli út 2-6.

4.5. Sponsor

KAQUN HUNGÁRIA Kereskedelmi Kft.

2144 Kerepes, szabadság út 102.

Authorised representative: Dr. Gyula Sebestyén, Scientific Counsellor

Semmelveis Medical University

1097 Budapest, Nagyvárad tér 2.

5. EXAMINATION AND CONTROL MATERIALS

5.1. Chemical and physical properties of the examination material

Name:	Kaqun water, for bathing
Manufacturer:	Kaqun Hungária Kft.
Delivered quantity:	2 x 1.5 l
Manufacturing number:	25/10/2010

CAS number: -

Number of analytical certificate:	Kerepes (2010/K/2192)
Number of microbiological inspection:	1-1298-2010
Colour:	water clear, colourless
Smell:	without smell
Storage conditions:	at room temperature
Safety regulations:	-
Expiry date:	08/10/2011

5.1.1. Stability examination

No stability examination was carried out for Kaqun water.

5.2. Control materials and solvent

5.2.1. Culture liquid

Name: Dulbecco’s Medium W/Pyruvate powder

Manufacturer: Gibco Invitrogen Corporation

Manufacturing number: 757533

Storage conditions: 2-8°C

Safety regulations:-

Expiry date: 30/04/2011

Name: DMEM, Dulbecco's Modified Eagly Medium 1X

Manufacturer: Gibco Invitrogen Corporation

Manufacturing number: 712334

Storage conditions: 2-8°C

Safety regulations:-

Expiry date: 31/12/2010

5.2.2. Positive control

Name: Dimethyl sulfoxide

Manufacturer: Sigma-Aldrich Kft.

Manufacturing number: BCBB 0540

CAS number: [67-68-5]

Storage conditions: at room temperature

Safety regulations: use protective gloves and glasses

Expiry date: 30/03/2014

5.3. Other materials used for the examination

5.3.1. Penicillin-streptomycin solution

Name: Penicillin Streptomycin (100x)

Manufacturer: PPA Laboratories GmbH

Manufacturing number: P01009-1954

Storage conditions: below -15°C

Safety regulations: use protective gloves

Expiry date: 31/08/2011

5.3.2. Serum

Name: Foetal beef serum (FBS EU Approved origin)

Manufacturer: Gibco Invitrogen Corporation

Manufacturing number: 41Q8095F

CAS number:-

Storage conditions: between -5 and -20°C

Safety regulations: use protective gloves

Expiry date: 31/05/2014

5.3.3. Trypsin solution

Name: Trypsin-EDTA (10X)

Manufacturer: Gibco Invitrogen Corporation

Manufacturing number: 695604

CAS number:-

Storage conditions: between -5 and -20°C

Safety regulations: use protective gloves

Expiry date: 30/04/2011

5.3.4. PBS solution

Name: PBS pH 7,4 W/O CAMG USA

Manufacturer: Gibco Invitrogen Corporation

Manufacturing number: 779745

CAS number:-

Storage conditions: between 15 and 30°C

Safety regulations:-

Expiry date: 31/05/2012

5.3.5. Isopropyl-alcohol

Manufacturer: Sigma-Aldrich Kft.

Manufacturing number: 078K0666

CAS number:[67-63-0]

Storage conditions: between 15 and 30°C, under nitrogen protective gas

Safety regulations: use protective gloves

Expiry date: 30/06/2011

5.3.6. MTT paint

Name: Thiazolyl Blue Tetrazolium Bromide

Manufacturer: Sigma-Aldrich Kft.

Manufacturing number: MKBC3383

CAS number: [298-93-1]

Storage conditions: between 2 and 8°C

Safety regulations: use protective gloves

Expiry date: 31/10/2012

5.3.7. Sodium-hydrogen-carbonate

Manufacturer: Sigma-Aldrich Kft.

Manufacturing number: BCBB 8363

CAS number: [144-55-8]

Storage conditions: between 15 and 30°C

Safety regulations:-

Expiry date: 28/02/2015

6. TEST SYSTEM

6.1. Description of the cell line

Human hepatocellular carcinoma (HepG2) cell line of epithelial origin was used for the examination. The code number of the used cell line is ATCC-HB-8065, Lot N: 58210525, place of origin: Manassas, VA 20110-2209 USA. The HepG2 is a permanent cell line. It was isolated from the hepatocellular carcinoma of a 15 year old boy. This cell line has the following characteristics: high level of morphologically differentiated state, non-tumorigenic, its chromosome number is 55. HepG2 cells secret plasma proteins such as albumin, transferrin, fibrinogen and plasminogen. HepG2 cells are propagated in MEM culture liquid modified by Dulbecco, which is supplemented before use by foetal beef serum with a final concentration of 10%, and also by antibiotics with penicillin final concentration of 10 U/ ml, and streptomycin final concentration of 10 µg/ml. The cell strain culture is stored in liquid nitrogen, an ampoule cell is taken from this store and before testing it is kept in continuous culture that is used until 15 passage numbers, then a new ampoule cell is taken. The cell with batch number 58210525/5 is used for the examination.

6.2. Grounds for selecting the test system

The citotoxicity examination may be carried out using both primary and permanent cell lines. However, we endeavoured to achieve examination conditions that are the most similar to use conditions. Therefore HepG2 cell line of human origin was chosen as the examination material is also for human use.

6.3. Checking the cell line

The used HepG2 cell line is checked once a year according to the following:

- optical density values of untreated control cells are measured

- the level of how free the cell is from mycoplasma was checked, the result was negative

7. METHOD

7.1. Citotoxicity examination

7.1.1. Brief description of the method

Live, metabolically active cells absorb 3-(4-5dimethylthiazol-2yl)-2,5-diphenyltetrazolium-bromide (MTT) paint, which is then reduced to colourful formazane salts by mytocondrial dehydrogenase enzymes. The quantity of the transformed colourful formazane salt is proportional to the number of live cells, and it is soluted from the cells by izopropanol, and

measured by colorimetric method. In the citotoxicity examination, 3000 cells were located per each hole of the 96-hole cell culture pot then following 24-hour incubation a 24-hour treatment was carried out. The citotoxicity examination was carried out with undiluted kaqun water.

7.2. Preparation of examination samples

7.2.1. DMEM culture liquid prepared with Kaqun water

1.9989 g was measured from the DMEM (Dulbecco's medium w/pyruvate; GIBCO Invitrogen Corporation, Lot N:757533) powder, then soluted in 200 ml of Kaqun water, and 0.7410 g sodium-hydrogen-carbonate was added (Sigma Aldrich Kft., Lot N:BCBB8363) and mixed until dissolved, then strained through a 0.22µm Millex filter to obtain a sterile state. 20 ml inactivated foetal beef serum (10 vol%; Gibco Invitrogen Corporation; Lot N:41Q8095F) and 200 µl penicillin/streptomycin solution (10 000 U/ml penicillin and 10 000 µg/ml

streptomycin; PAA Laboratories GmbH; LotN: P01009-1954) was added to the sterile culture liquid prepared this way.

Undiluted kaqun water and this method of preparing the examination material were chosen so that we can provide the optimal quantity of nutrient needed for the growth of the cells and can investigate the 100% concentration of the examination material at the same time. In addition to this we chose this highest value as it is used in practice in this form as well.

7.2.2. DMEM culture liquid prepared with ultra-pure water

1.9975 g was measured from the DMEM (Dulbecco's medium w/pyruvate; GIBCO Invitrogen Corporation, Lot N:757533) powder, then soluted in 200 ml ultra-pure water, and 0.7405 g sodium-hydrogen-carbonate was added (Sigma Aldrich Kft., Lot N:BCBB8363) and mixed until dissolved, then strained through a 0.22µm Millex filter to obtain a sterile state. 20 ml inactivated foetal beef serum (10 vol%; Gibco Invitrogen Corporation; Lot N:41Q8095F) and 200 µl penicillin/streptomycin solution (10 000 U/ml penicillin and 10 000 µg/ml streptomycin; PAA Laboratories GmbH; LotN: P01009-1954) was added to the sterile culture liquid prepared this way.

7.2.3. Preparing positive control solution

2.5 ml DMSO (Sigma-Aldrich Kft.; Lot No: BCBB0540 was added to 50 ml DMEM (LG) W/NA PYR. (Gibco Invitrogen Corporation; Lot No: 712334) culture liquid.

7.3. Placing the examination samples in 96-hole tissue culturing pot

Column number	Description of the sample
1	Positive control (5% DMSO)
2	
3	DMEM culture liquid prepared with Kaqun water
4	
5	
6	
7	DMEM culture liquid prepared with ultra-pure water
8	
9	DMEM culture liquid (Manufacturing number: 712334)
10	Untreated control
11	Cell-free control
12	

8. Measuring the citotoxicity examination

Optical density was measured by Multiskan FC photometer (570nm/620nm). Optical density values were evaluated by Multiskan FC 2.5.1. program, and average and standard deviation were calculated by concentrations.

9. Evaluating the citotoxicity examination

9.1. Negative result

Negative result means that in the given experimental conditions the examination material does not significantly reduce the rate of viable cells compared to the untreated control.

9.2. Positive result

The examination material is citotoxic if it reduces the percentage rate of viable cells significantly in a dose-dependant way, reproducibly, and at one or more concentration levels compared to the untreated control.

10. Statistical evaluation

The data were evaluated by the Dunnett test in the one-way ANOVA statistical program running in the Graphpad computer program. The untreated control group was compared to the treated group averages.

11. Results of the citotoxicity examination

11.1. Summary table of optical density values measured at 570nm/620 nm of Plates 1 and 2

DMEM control Columns A09-H09;A10-H10		DMEM with Kaqun water Columns A03-04-05-06;H03-04-05-06				DMEM with ultra-pure water Columns A07-H07;A08-H08		Positive control A01-H01;A02-H02	
0.147	0.167	0.135	0.159	0.150	0.139	0.163	0.158	0.044	0.048
0.184	0.171	0.204	0.181	0.162	0.194	0.195	0.180	0.052	0.044
0.173	0.196	0.206	0.216	0.186	0.204	0.226	0.178	0.057	0.045
0.228	0.185	0.222	0.216	0.212	0.212	0.196	0.203	0.052	0.050
0.226	0.239	0.213	0.237	0.200	0.178	0.190	0.213	0.046	0.046
0.192	0.199	0.210	0.291	0.221	0.220	0.215	0.239	0.044	0.039
0.201	0.232	0.202	0.205	0.237	0.202	0.233	0.205	0.059	0.045
0.202	0.193	0.169	0.194	0.165	0.147	0.156	0.162	0.062	0.050
0.108	0.141	0.144	0.202	0.217	0.152	0.204	0.155	0.051	0.050
0.132	0.124	0.174	0.223	0.210	0.195	0.186	0.156	0.051	0.038
0.187	0.195	0.189	0.225	0.206	0.209	0.195	0.194	0.056	0.043
0.202	0.167	0.188	0.199	0.219	0.177	0.161	0.181	0.051	0.043
0.143	0.136	0.186	0.171	0.180	0.161	0.178	0.196	0.048	0.039
0.167	0.136	0.181	0.183	0.168	0.202	0.172	0.194	0.053	0.045
0.195	0.152	0.154	0.188	0.195	0.172	0.196	0.164	0.043	0.036
0.103	0.109	0.134	0.141	0.128	0.152	0.142	0.140	0.044	0.036
Average:0.173		Average:0.189				Average:0.185		Average:0.047	
Std		Std deviation:0.024				Std		Std	

deviation:0.031		deviation:0.021	045deviation:0.005

11.2. Summarizing evaluation of citotoxicity examination

	Measurement results		Paint reduction %	Evaluation
	Average	Std deviation		
Positive control	0.047	0.005	27.16	Positive
DMEM with Kaqun water	0.189	0.024	109.2	Negative
DMEM with ultra-pure water	0.185	0.0.21	106.9	Negative
DMEM control	0.173	0.031	100	Negative

12. Summarizing the results

In the given experimental conditions Kaqun water did not reduce the rate of viable cells compared to the untreated control.

Kaqun water does not have any citotoxic effect.

13. ARCHIVING

Examination specific documentation (Examination Plan, raw data) and non-examination specific documentation will be retained for 15 years, whereas examination material will be retained for expiry time plus 1 year. The Closing Report will not be scrapped. Archiving will take place at Molecular and Cell Biological Department of the National Institute of Chemical Safety (Budapest, Gyáli út 2-6. Building C, Groundfloor). After the given time, before destroying all materials shall be offered to the Sponsor for retaining.

Budapest, 20/12/2010

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Dr. Zsuzsanna Kocsis

Annex 1

Citotoxicity examination of Kaqun water in HepG2 cells

Micro plate 1

Optical density values (570nm/620nm)

Columns 01-02: Positive control (5% DMSO)

Columns 03-06: DMEM prepared with Kaqun water

Columns 07-08: DMEM prepared with ultra-pure water

Columns 09-10: DMEM culture liquid

Columns 11-12: blind, technical control (cell-free sample)

From A to H: data of the individual parallel samples

	01	02	03	04	05	06	07	08	09	10	11	12
A	0.044	0.048	0.135	0.159	0.150	0.139	0.163	0.158	0.147	0.167	0.035	0.033
B	0.052	0.044	0.204	0.181	0.162	0.194	0.195	0.180	0.184	0.171	0.042	0.033
C	0.057	0.045	0.206	0.216	0.186	0.204	0.226	0.178	0.173	0.196	0.038	0.030
D	0.052	0.050	0.222	0.216	0.212	0.212	0.196	0.203	0.228	0.185	0.024	0.031
E	0.046	0.046	0.213	0.237	0.200	0.178	0.190	0.213	0.226	0.239	0.033	0.027
F	0.044	0.039	0.210	0.291	0.221	0.220	0.215	0.239	0.192	0.199	0.029	0.026
G	0.059	0.045	0.202	0.205	0.237	0.202	0.233	0.205	0.201	0.232	0.023	0.022
H	0.062	0.050	0.169	0.194	0.165	0.147	0.156	0.162	0.202	0.193	0.022	0.025

Concentration	Column	Description of sample	Standard deviation	Average	CV%
Positive control 5% DMSO	A01	5% DMSO	0.006	0.049	12.67
Positive control 5% DMSO	A02	5% DMSO			
DMEM with Kaqun water	A03	Kaqun	0.033	0.196	16.56
DMEM with Kaqun	A04	Kaqun			

water					
DMEM with Kaqun water	A05	Kaqun			
DMEM with Kaqun water	A06	Kaqun			
DMEM with ultra-pure water	A07	Ultra-pure water	0.027	0.195	13.74
DMEM with ultra-pure water	A08	Ultra-pure water			
DMEM culture liquid	A09	DMEM	0.026	0.196	13.02
DMEM culture liquid	A10	DMEM			
Cell-free control	A11	Cell-free control	0.008	0.031	24.26
Cell-free control	A12	Cell-free control	0.004	0.028	14.68

Number of examination: 02-CTOX-10

Annex 2

Citotoxicity examination of Kaqun water in HepG2 cells

Micro plate 2

Optical density values (570nm/620nm)

Columns 01-02: Positive control (5% DMSO)

Columns 03-06: DMEM prepared with Kaqun water

Columns 07-08: DMEM prepared with ultra-pure water

Columns 09-10: DMEM culture liquid

Columns 11-12: cell-free sample

From A to H: data of the individual parallel samples

	01	02	03	04	05	06	07	08	09	10	11	12
A	0.051	0.050	0.144	0.202	0.217	0.152	0.204	0.155	0.108	0.141	0.033	0.039
B	0.051	0.038	0.174	0.223	0.210	0.195	0.186	0.156	0.132	0.124	0.031	0.024
C	0.056	0.043	0.189	0.225	0.206	0.209	0.195	0.194	0.187	0.195	0.023	0.018
D	0.051	0.043	0.188	0.199	0.219	0.177	0.161	0.181	0.202	0.167	0.020	0.018
E	0.048	0.039	0.186	0.171	0.180	0.161	0.178	0.196	0.143	0.136	0.019	0.019
F	0.053	0.045	0.181	0.183	0.168	0.202	0.172	0.194	0.167	0.136	0.028	0.020
G	0.043	0.036	0.154	0.188	0.195	0.172	0.196	0.164	0.195	0.152	0.025	0.017
H	0.044	0.036	0.134	0.141	0.128	0.152	0.142	0.140	0.103	0.109	0.020	0.018

Concentration	Column	Description of sample	Standard deviation	Average	CV%
Positive control 5% DMSO	A01	5% DMSO	0.006	0.045	13.65
Positive control 5% DMSO	A02	5% DMSO			
DMEM with Kaqun water	A03	Kaqun	0.026	0.182	14.55
DMEM with Kaqun water	A04	Kaqun			
DMEM with Kaqun water	A05	Kaqun			
DMEM with Kaqun water	A06	Kaqun			
DMEM with ultra-pure water	A07	Ultra-pure water	0.021	0.176	11.75
DMEM with ultra-pure water	A08	Ultra-pure water			
DMEM culture liquid	A09	DMEM	0.032	0.150	21.67
DMEM culture liquid	A10	DMEM			
Cell-free control	A11	Cell-free control	0.005	0.025	21.27
Cell-free control	A12	Cell-free control	0.007	0.021	33.88