

HEPATOTOXICITY – WEB-TOOL.

The purpose of developed Web-tool is the predictive analysis (extrapolation) of toxicity profile for substances released from a biomaterial during its biodegradation.

The developed software provides possibility to predict the level of toxicity of soluble factors, released both by ordinary dissolution and by biodegradation of the material with the participation of biological factors (for example, pericellular proteolysis).

An obvious prerequisite for the model construction is the assumption that biomaterials with obvious high toxicity (for example, $LD_{50} < 10 \text{ mg / ml}$, $t < 12 \text{ h}$) are to be excluded from the following testing already at the stage of preparing implantable materials using standard in vitro cytotoxic tests.

At the same time, for biomaterials with initially low and not obvious toxicity we preliminarily implement the analysis that should uncover whether significant difference with the control (obviously non-toxic) substance is present. For this aim some programs from “tox-screen” part of this tool should be applied. For example the analysis can be carried out using program “Proliferation”, and further step using program “Hepatotoxicity test.”

The advantage of this method is that it allows to identify / predict effects that are impossible or difficult to register using standard methods of toxicological analysis in vitro and even in vivo: for example, if the cumulative toxic effect of substances, released inside recipient organism from a biomaterial, is so negligible that cell death in significant / reliable values will be observed only after hours or even days for cell culture (that conventional experimental methods can not reveal or estimate properly). In this situation the program gives possibility to predict the $LD_{50}(30)$, as well as to predict the concentration of a substance that can cause a toxic effect (it opens possibilities for prediction of hepatotoxic and carcinogenic effects at the level of the recipient organism too).

In this program, we partially used the approach developed for estimation of hepatotoxicity of some pharmacological substances in order to calculate the kinetics of cell death. This approach describes the cell death under the influence of metabolites produced from the primary (test) substance (drug) processed by hepatocytes.

The programs provides possibility to define specific cell death pattern typical to the substances. At the first phase, the coefficient of natural cell death can be calculated (using parameters of control culture), after that at the second phase, parameters of cell death under the influence of tested substance can be defined. Additionally, if the initial concentration of the toxin is determined by any chemical (photometric, etc.) technique, the LC_{50} can be easily calculated (however, even in the case of unknown initial concentration some function of this parameter still can be calculated). The final task of elaborated program is to analyze the experimental data using a suitable mathematical model describing the toxic effect in order to determine the parameters of the model and extrapolate the toxic effect for long period for evaluation of LC_{50} or its equivalent (for example, specific coefficient defined by the area of tested biomaterial seeded with target cells). Then it is possible to recalculate the toxic effect for other conditions (in vivo + other surface area of the biomaterial that releases the toxin).

Since the range of measurements using the applied model is limited by a few values, we offer here several options to demonstrate how hepatocytes can process a substance to form toxic metabolites, that in its

turn affects the viability of target cells (see please attached files with initial values for paracetamolum, diclofenak etc.)

EXAMPLES.

The values should be inserted manually (we will adapt data input by files during a few weeks).

Testing species concentration*,uM - "T"

Rate of cell natural death**, 1/h - "gamma"

Reference time for LC50 evaluation,h - "beta"

Concentrations in % to initial - "Cexp" – experimental values

Acetaminophen :

```
"T": 5000.000,  
"gamma": 5.300e-02,  
"beta": 1.656e-02,  
"mu": 2.696e-05,  
"t_ref": 1.50,  
"LC50":3.476e-308,  
"Cexp":  
100.000,100.000,100.000,94.200,78.100,56.900,43.100,32.800,27.000,16.100, 0.000, 0.000, 0.000
```

Verapamil

```
"T": 100.000,  
"gamma": 5.300e-02,  
"beta": 2.117e-02,  
"mu": 2.982e-03,  
"t_ref": 1.50,  
"LC50":3.476e-308,  
"Cexp":  
[  
 100.000,99.300,83.800,71.300,54.400,35.300,13.200, 0.000, 0.000, 0.000, 0.000, 0.000]
```

Diclofenac

```
"T": 300.000,  
"gamma": 5.300e-02,  
"beta": 4.117e-02,  
"mu": 1.093e-03,  
"t_ref": 1.50,  
"LC50":3.476e-308,  
"Cexp": [ 100.000,100.000,93.400,83.800,54.400, 5.880, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000,
```

The “HEPATOTOXICITY” program module has been initially elaborated for simulation of toxicity processes in hepatocellular culture in vitro. The module provides minimal calculation error and gives possibility end-user to estimate range of parameters characterising material biocompatibility according to cytotoxic tests. Models have been further adapted for evaluation of kinetic toxic parameters for cell of other species. The limitation of this program set refer to the strict requirements to experimental design and parameters of input data.

All module programs have a similar interface. It includes input of some conditions for the analysis of input data, as well as experimental data obtained at specified time points of measurement.

Model of cyto- (hepato-) toxicity under direct toxin influence (Hep1T)

Set up a cytotoxicity experiment in which the concentration of the toxin will remain constant during the test. If cell proliferation can be neglected, then in the first approximation the rate of cell death can be considered proportional to the product of the concentration of target cells, the concentration of insult and a parameter characterizing the toxic properties, for example, the rate of degradation of the polymeric material.

The main page of service Hep1T has the form shown in **Figure 1**. Here it is assumed that the concentration of the toxin is constant during the test. User should fill the forms manually changing the pre-existing values specified initially in the form by default. After that “Submit” button should be pressed to start the program. Results shows the results of inverse problems solution (**Figure 2**). For the case of values set by default the experimental data were simulated for the case of exponential dependence of cell concentration on time with the measurement error of 3%. The exact value of the effective toxicity parameter was set as 0.1 h^{-1} . The inverse problem was to determine the parameter $\delta = \delta_T T$, by which the model describes dependence of cell concentration on time that approximates the experimental data in the best manner. The decision is showed in form of the theoretical curve for this dependence with the found value of the toxicity parameter δ . As can be seen from the figure the hepatotoxicity parameter was determined with an error of 1.2%, which indicates the high efficiency of the algorithm for fitting.

Enter data

Rate of cell natural death*, 1/h	0.001
Time 0	100.0
Time 30	101.0
Time 60	88.89
Time 90	89.47
Time 120	82.21
Time 150	77.55
Time 180	75.00
Time 210	71.09
Time 240	66.27
Time 270	63.58
Time 300	61.22
Time 330	56.92
Time 360	53.46

Submit

*To calculate the rate of natural cell death, set its value to - δ (in case of toxin absence!)

Processing about 3 s

Figure 1. Main input form of “model of cyto- (hepato-) toxicity under direct toxin influence”.

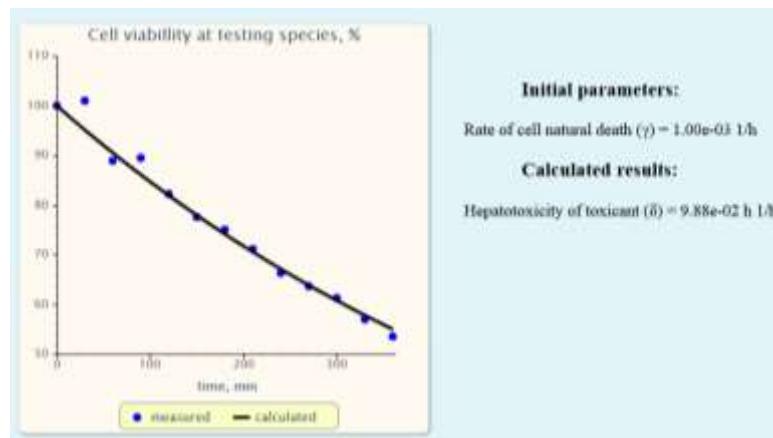


Figure 2. The example of a simulation using the values outlined in the Figure 44.

Evaluation of the rate of natural cell death

The rate of natural cell death can be evaluated using service mathematicaly. Input data for such analysis and its result are shown in **Figure 3**. To define the conditions when only the rate of natural cell death is calculated, the value of this parameter should be set as “1”.

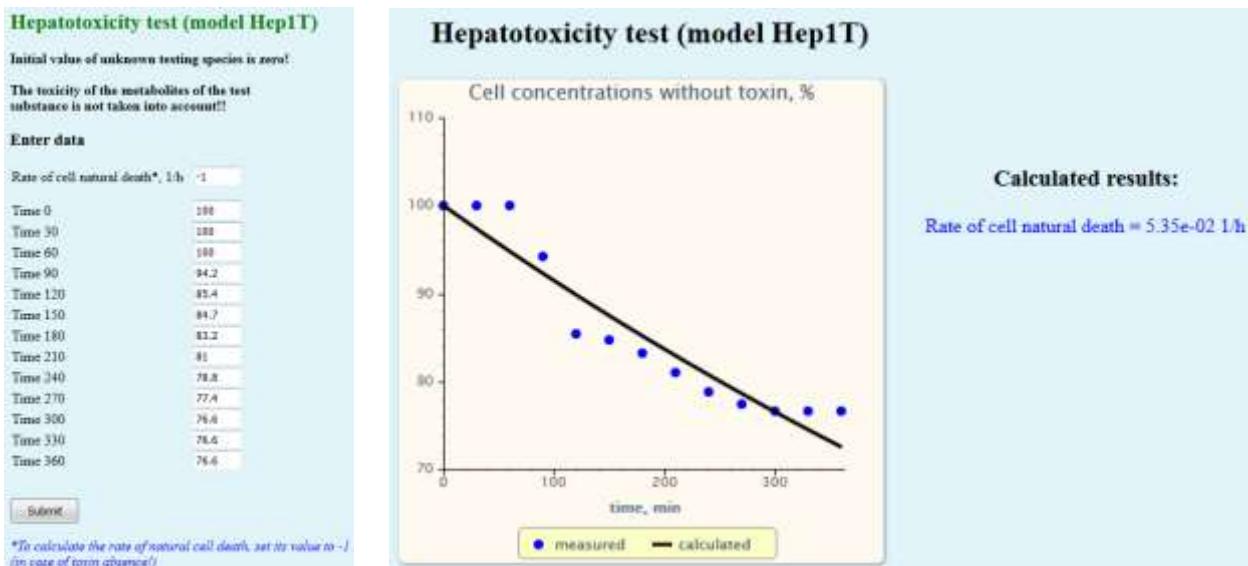


Figure 3. Initial data to determine the rate of natural cell death and calculated results

Model of cyto- (hepato-) toxicity under secondary (metabolite) toxin influence (HEP1M)

The main page of this program has the form shown by **Figure 4**. Here it is assumed that the concentration of the test substance is constant during the test. Toxicant is a product of the cellular metabolism of the test substance. If the concentration of the test substance is unknown, the parameter of “effective toxicity” is calculated (it includes two coefficients: the concentration of the test substance and the toxicity level). User should fill the forms manually, changing the pre-existing values specified initially in the form (tables) for the illustrative purposes. The figure shows an example of the experimental data for testing of substance with known concentration. Button “Submit” should be used to run the program.

Input data

Concentration of the test substance*, μM	5000
Rate of cell natural death**, 1/h	0.053
Reference time for LC50 evaluation,h	1.5
Time 0	100.0
Time 30	100.0
Time 60	100.0
Time 90	94.2
Time 120	81.1
Time 150	56.9
Time 180	43.1
Time 210	32.8
Time 240	27.0
Time 270	16.1
Time 300	0
Time 330	0
Time 360	0

* If the concentration of the test substance is not known, then a value of 1 can be set.
Then the product of this unknown concentration and toxicity parameter will be calculated.

** To find the rate of cell natural death set zero for the concentration of the test substance.
Too much of the rate of natural cell death can lead to an error in the calculation of toxicity parameters!

Figure 4. Input form of model of cyto- (hepato-) toxicity under secondary (metabolite) toxin influence

In 2-5 seconds from the moment of program start, the result of calculation will be displayed on the user's computer in form of the curves, showing the dependence of cell concentration on the time. The input experimental data and calculated parameters of this model, β and μ , as well as the parameter LC50 denoting the concentration of the test substance at which half of the cells will survive after a specified period (reference time for LC50 evaluation) will be displayed **Figure 5**.

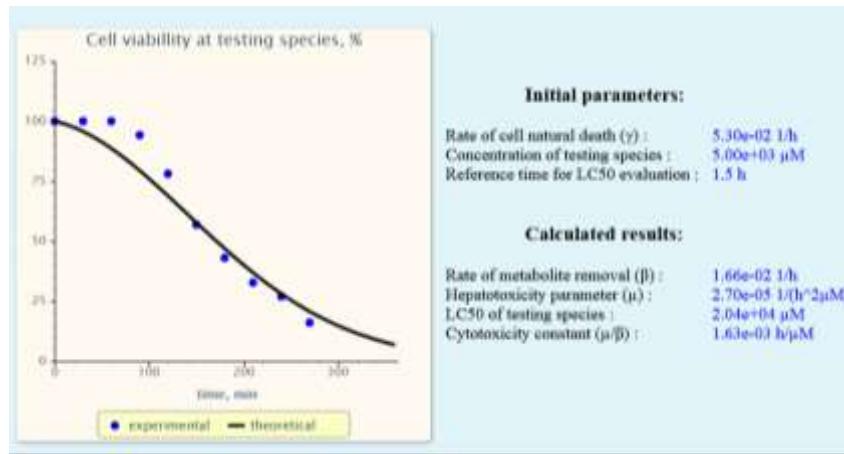


Figure 5. Example of calculated results for the model of cyto- (hepato-) toxicity under secondary (metabolite) toxin influence.

Evaluation of hepatotoxicity of diclofenac metabolite

We estimate the hepatotoxicity of the diclofenac (10mM) as substance with mostly metabolic (secondary) toxicity. The magnitude of the rate of natural death of hepatocytes (under experimental conditions without a test substance) is obtained in the previous example, i.e. 0.053 1 / h. The input data for the analysis and its result are shown in **Figure 6**.

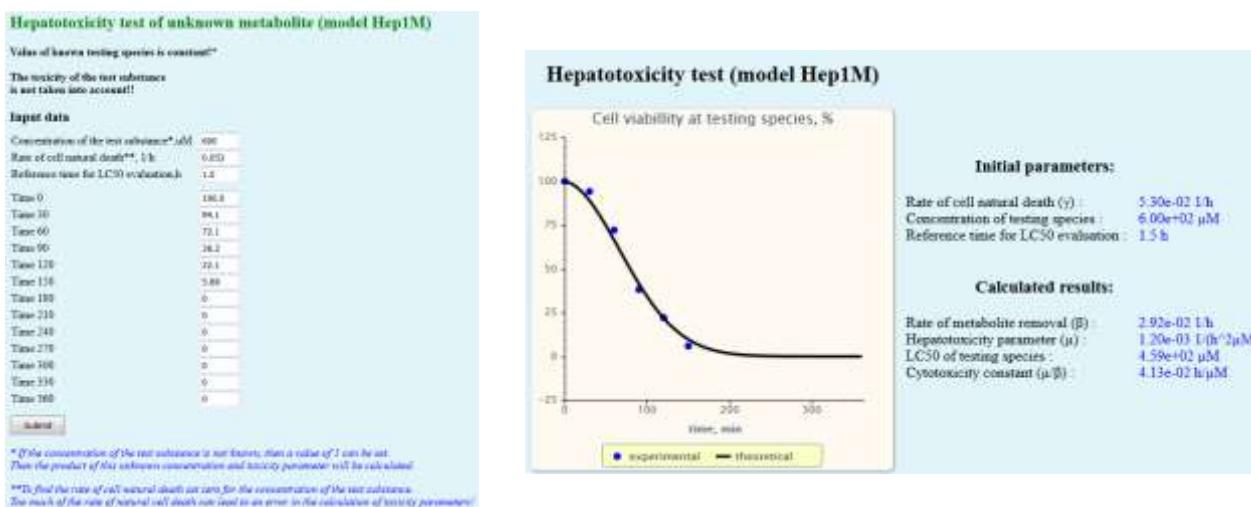


Figure 6. Initial data to determine the cytotoxicity of Diclofenac and calculated results.

Model of cyto- (hepato-) toxicity depends on toxin degradation rate (Hep2)

The input form of service Hep2 has shown in **Figure 7**. Here it is assumed that the initial concentration of toxicant is zero and increases with time as a result of biodegradation of a polymer substrate. In this case, initially only one parameter is set — the rate of natural cell death γ , as well as the set of values of the relative cell concentration at different times with a step multiple of 30. User should fill the forms manually changing the pre-existing values specified initially in the form for illustrative purposes. After that “Submit” button should be pressed to start the program. If the user wants to determine the rate of natural cell death for his experimental data (if it is unknown) he should insert the number «1» in field «Rate of cell natural death» after entering relative concentration value into the table (form). The program will then independently calculate the best fitted value for this rate. It should be taken into account that the specified positive value of this rate cannot be greater than the actual effective cell death rate corresponding to the experimental data entered when testing the toxin. The result (**Figure 8**) displays the

"characteristic" time of biodegradation of material. The time after which the fraction of the remaining cells will decrease to 50% from the original quantity is also estimated.

Enter data

Rate of cell natural death*, 1/h	0.001
Time 0	100.0
Time 30	100.8
Time 60	94.66
Time 90	90.94
Time 120	82.92
Time 150	74.74
Time 180	66.63
Time 210	57.91
Time 240	49.18
Time 270	41.47
Time 300	34.45
Time 330	27.69
Time 360	22.29

Submit

*To calculate the rate of natural cell death, set its value to -1 (in the absence of toxin!)

Processing about 3 s

Figure 7. Input form of model of cyto- (hepato-) toxicity depends on toxin degradation rate.

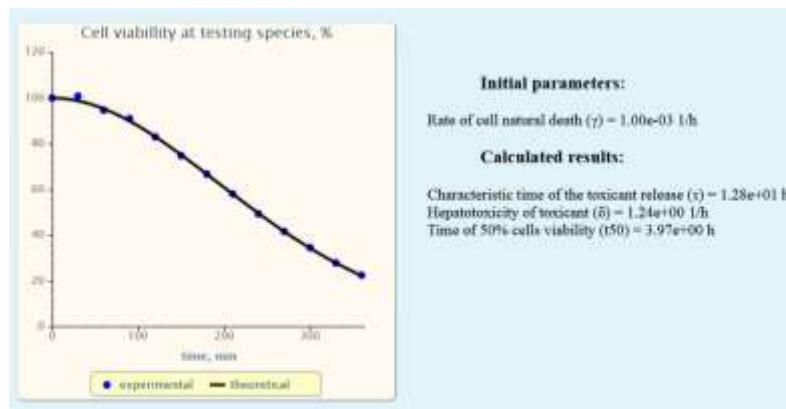


Figure 8. Example of calculated results for the model cyto- (hepato-) toxicity depends on toxin degradation rate

Evaluation of the cytotoxicity of the substances released during biomaterial degradation.

Evaluation of the cytotoxicity of substances released during biodegradation of a polymeric material is demonstrated by figures below. The direct and secondary (metabolite-associated) toxicity of a biodegradable polymeric material was simulated. In both cases, the rate of natural death of hepatocytes was set to zero. The "characteristic" time of biodegradation of the polymer was set to 72 hours. In the case of direct toxicity, the effective cytotoxicity parameter δ was taken equal to 0.1 h⁻¹. in other case cytotoxicity corresponded to the value δ = 10 h⁻¹. **Figure 9** and **Figure 10** outlined results of corresponding experiments. Simulated measurement errors are 0.001% and 0.1%, respectively.

These figures show that, with a sufficiently high accuracy of cell concentration measurements, the Hep2 service allows the evaluation of the toxic properties of a biodegradable polymeric material. The increase of the duration of the toxicological test and the number of measured concentrations, as far as, reducing the time step between the individual measurements can be applied to reduce the error of calculation.

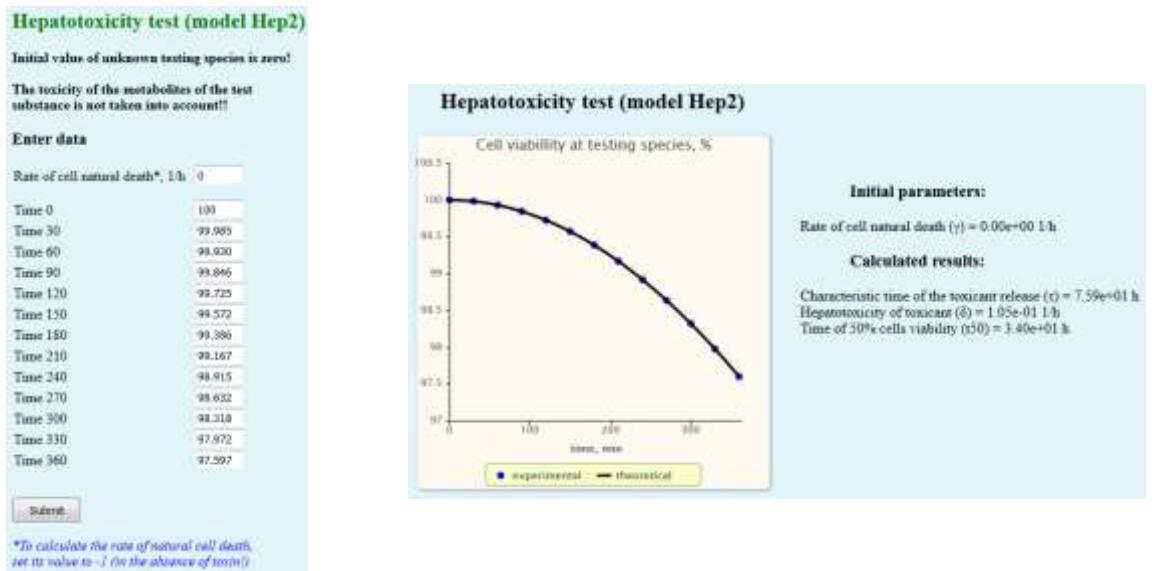


Figure 9. Initial data of material cyto-toxicity (direct toxicity) with fixed degradation rate and calculated results.

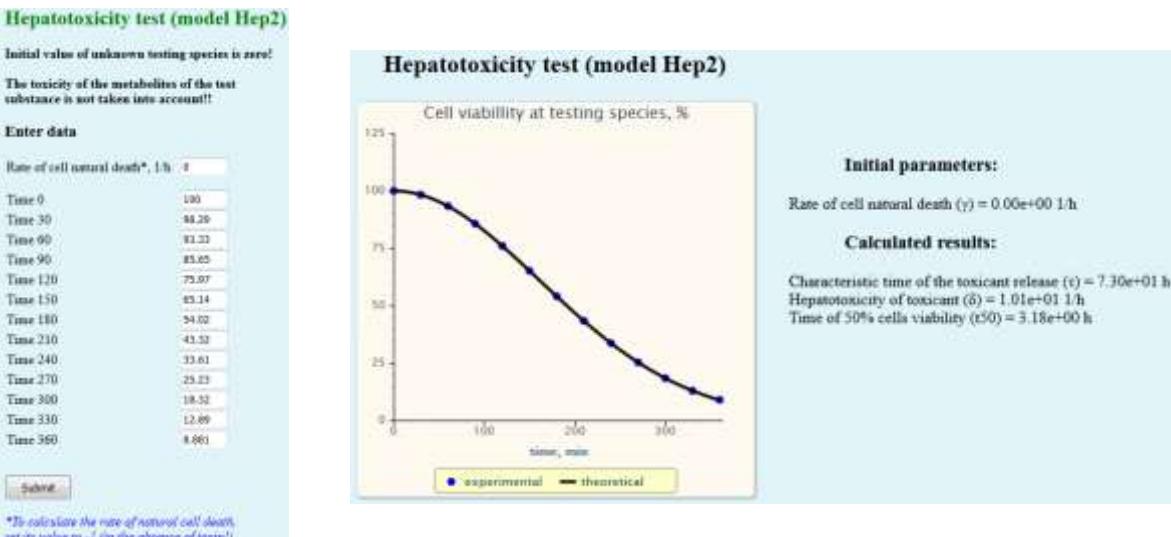


Figure 10. Initial data of material cyto-toxicity (secondary (metabolite-associated) toxicity) with fixed degradation rate and calculated results.