In vitro Cytotoxicity
of Manorapid Synergy/Perfume Fresh PH 799 982
vis-à-vis L929 Cells

The specimens made available:

Reference solution 1: 0.234 % Tween 20 and 99.766 % water of 14/09/2004
Reference solution 2: purified water of 14/09/2004
Test solution 1: 0,234 % Tween 20, 0.156 % Perf. Fresh 799982 and 99.688 % purified water of 14/09/2004 were subjected to in vitro cytotoxicity testing as per DIN EN ISO 10993-5:1999 (Biological Evaluation of Medicinal Products, Part 5. Testing for cytotoxicity: in vitro methods).

The test cells used were mouse fibroblasts (L929 cells, ATCC CCL1).

To test for in vitro cytotoxicity on the basis of Neutral Red (NR) absorption as well as with the MTT test, test solution 1 was combined with a 2-fold concentrated culture medium in order to obtain the final perfume concentration that is found in the product as used. Since it is not possible to dissolve the perfume directly in water, the solubilizer Tween 20 was used in an optimal concentration. Reference solution 1 (only solubilizer Tween 20 in purified water) and reference solution 2 (only purified water) were also mixed with 2-fold concentrated culture medium. From the resultant 50 % test solutions in the culture medium, further dilutions of 25 %, 12.5 % and 6.25 % (referred to the respective baseline test preparation) were used for in vitro cytotoxicity testing.
The time during which the various test concentrations were in contact with the test cells (contact time) was 24 h.
All test solutions and dilutions available were subjected to triplicate testing with corresponding extraction media.
As per its definition, the mean absorption of the medium control batch is deemed to be equivalent to 100 % vitality and vitality of the cells in the test batches is determined in relation to this.

The Neutral Red (NR) and MTT Method

With the neutral red method it is possible to detect both the reduction in cell proliferation as well as the vitality of cells. It is based on the absorption of the supravital pigment neutral rot (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) and its accumulation in the lysosomes of living cells. Damage to the cell and/or lysosomal membranes, mediated by toxic substances, result

in no pigment being absorbed. The stored neural red can be eluted from the lysosomes of living cells on using an acetic acid ethanol solution. Staining of the supernatant is evaluated with spectrophotometry, with absorption being a direct measure of cell vitality.

Determination of cell vitality with the MTT method is based on the fact that living cells convert, with the help of mitochondrial succinate dehydrogenase, the yellow-stained tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) into a blue, insoluble formazan derivate and store it in the mitochondria. The precipitated and deposited formazan derivate is then eluted with acidic 2-propanol. Staining of the supernatant is evaluated with spectrophotometry, with absorption being a direct measure of cell vitality.

Tab. 1

Vitality [%] of L929 cells in the in vitro cytotoxicity test after 24 h contact with test- preparation dilutions in the culture medium based on the NR and MTT method

Test preparation in the culture medium	Final concentration of Perfume or Tween 20	Vitality [%]	
		MTT Test	NR Test
50 % Reference solution 1	0.117 % Tween 20	55.5	13.5
25 % Reference solution 1	0.0585 % Tween 20	103.6	88.5
12.5 % Reference solution 1	0.02925 % Tween 20	100.6	93.6
6.25 % Reference solution 1	0.014625 % Tween 20	101.4	95.0
50 % Reference solution 2	-	101.6	98.2
25 % Reference solution 2	-	101.9	96.9
12.5 % Reference solution 2	-	98.8	99.4
6.25 % Reference solution 2	-	97.2	98.6
50 % Test solution 1	0.117 % Tween 20 / 0.078 % Perfume	3.7	0.6
25 % Test solution 1	0.0585 % Tween 20 / 0.039 % Perfume	67.1	58.7
12.5 % Test solution 1	0.02925 % Tween 20 / 0.0195 % Perfume	97.2	96.7
6.25 % Test solution 1	0.014625 % Tween 20 / 0.00975 % Perfume	101.6	101.2

As expected, reference solution 2 (purified water) contains no cytotoxic active substances.

The solubilizer Tween 20 in reference solution 2 generates a cytotoxic effect in a final concentration of 0.117 %. Only in a concentration of 0.0585 % Tween 20 is the cytotoxic effect vis-à-vis L929 cells negligible.

Without doubt the addition of perfume potentiates the cytotoxic effect of Tween 20. Since Tween 20 still shows a cytotoxic reaction in a final concentration of 0.117 %, an additive effect cannot be