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ANALYSIS REPORT

(Industrial Technical Support Service)

Report no : B.14.2.TBT.5.01.13.00-181.06.03.126/3451

Report date : **05.03.2012**

Requested by : NORMMED MED. VE MAK. SAN. TIC. LTD. STI.

Address : IVEDIK ORG. SAN. AGAC ISLERI SAN. SIT. 521. SOK. NO: 43
YENIMAHALLE, ANKARA
PHONE: 0 312 3956184
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Subject : CYTOTOXICITY, IRRITATION, ACUTE SYSTEMIC TOXICITY, SENSITIZATION TEST PERFORMED FOR "SPINAL IMPLANT" WITHIN THE SCOPE OF BIOCOMPATIBILITY TESTS

The results contained in this report belongs to the examined sample only.

Approved by

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Assoc. Prof. Dr. Fatima YUCEL
GMBE Industrial Services Supervisor

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Report no	: B.14.2.TBT.5.01.13.00-181.06.03.126/**3451**		
Requested by	: NORMMED MED. VE MAK. SAN. TIC. LTD. STI.		
Address	: IVEDIK ORG. SAN. AGAC ISLERI SAN. SIT. 521. SOK. NO: 43 YENIMAHALLE, ANKARA		
Sample	: One sample	**Expiration date**	: -
Number of samples	: 150 ea.	**Production Date**	: -
Supply manner of the sample	: It was supplied in 5 packages containing 30 ea. sterile samples.	**Institute sample record no**	: 12/44-GMBE
Condition of the sample at the time of acceptance	: It was supplied in 5 packages containing 30 ea. sterile samples.	**Date and time of acceptance**	: 01.02.2012
		Date of analysis	: 06.02.2012 - 02.03.2012

Witness sample info:	( ) Return to the customer	(x) Witness sample is available	( ) Witness sample was not taken.

1- Samples

The analyses of the product which is defined as "spinal implant" were performed in order to carry out the cytotoxicity, irritation, acute systemic toxicity and sensitization tests within the scope of the biocompatibility analyses.

Sample	Characteristic	Item
Spinal Implant	The product is spinal implant which is made from titanium and coated by silver and is called as spinal implant. The test samples were prepared in samples having the length of 10 mm, the width of 0,4 mm and thickness of 0,3 mm.	150

Table 1: Tested sample.

Remarks:

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2- Irritation Test

The irritation tests were performed by taking into account the standards "ISO 10993-10 Tests for irritation and delayed-type hypersensitivity, ISO 10993-2 Animal welfare requirements and ISO-10993-12 Sample preparation and reference materials"

The irritation tests of the samples were tried on the female New Zeland rabbits at the weight of not less than 2 kg. As specified in the document entitled ISO 10993-10, the tests were performed by applying the material to be tested directly onto the skin. For this, the administration plan applied on the experimental animals is shown in the Figure 1.

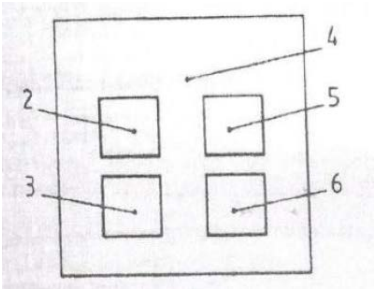

Figure 1. 2; test area, 3; negative control area, 5; test area, 6; positive control, 4; head part of the experimental animal.

Positive Control

90% lactic acid (1 ml) which had been previously known to have skin irritating effect was used as positive control (6).

Negative Control

Stainless steel which had been previously known not to have skin irritating effect was used as negative control (3).

After the experimental animals were shaved enough to provide sufficient administration area, the samples were applied as shown in the Figure 1. After closing the samples with a sterile gauze dressing, the entire administration area was wrapped with elastic bandage. As stated in ISO 10993-10, the samples to be tested were applied to the area for 4 hours. At the end of this period, the bandages were opened, the samples were taken and the administration areas were marked. Afterwards, the experimental areas were observed at the 1st, 24th, 48th and 72nd hours and the samples were evaluated in consideration of the criteria set forth in the Table 2. The evaluation results required to be given according to the scores obtained are submitted in the Table 3.

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Reaction	Score
Redness and scar formation	
No redness	0
Very slight redness (hardly noticable)	1
Visibly redness	2
Medium redness	3
Serious redness and scar formation	4
Oedema	
No oedema	0
Very slight oedema (hardly noticable)	1
Visibly oedema	2
Medium oedema (approximately 1 mm)	3
Serious oedema (larger than 1 mm)	4
Total possible score for irritation	8
Other undesired changes that might be observed on the skin should be also recorded and reported.	

Table 2. Evaluation criteria and scoring.

Average Score	Evaluation Category
0 - 0,4	Minor
0,5 -1,9	Slight
2 - 4,9	Medium
5 - 8	Advance

Table 3. Evaluation of the scoring results.

Remarks:

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Report no	: B.14.2.TBT.5.01.13.00-181.06.03.126/**3451**

Observation Results

The values and average score values obtained as a result of the examination made with Binoculer Lopus (3X) are submitted in the Table 4 and Table 5, respectively.

Samples	Area*	Observation (Hour)							
		Redness				Oedema			
		1.	24	48	72	1	24	48	72
Spinal Implant	*Left Front Area*	0	0	0	0	0	0	0	0
	Right Front Area	0	0	0	0	0	0	0	0
Positive Control	*Right Rear Area*	3	3	3	3	3	3	3	3
Negative Control	*Left Rear Area*	0	0	0	0	0	0	0	0

*Points out the administration areas clarified in the Figure 1.

Table 4. Evaluation results.

Samples	Area*	Redness	Oedema
Spinal Implant	*Left Front Area*	0	0
	Right Front Area	0	0
Positive Control	*Right Rear Area*	3	3
Negative Control	*Left Rear Area*	0	0

*Points out the administration areas clarified in the Figure 1.

Table 5. Average score values.

Result

As stated for the tested product, after four different observations were made (Table 4), the values obtained were averaged and the average score was obtained (Table 5). In the observations made for the tested samples, any oedema or redness was not observed. In line with the results obtained, **it was found that** the tested product **has not irritating characteristic** in accordance with the protocol and evaluation criteria specified in the document ISO 10993-10.

Remarks:

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3- Acute Systemic Toxicity Test

The Acute Systemic Toxicity Tests were performed in accordance with the protocols "ISO 10993-11 Biological evaluation of medical device - systemic toxicity, ISO 10993-2 Biological evaluation of medical devices - Part 2: Animal welfare requirements and ISO 10993-12 Biological evaluation of medical devices - Part 12: Sample preparation".

The test protocol followed in accordance with the specified standards is based on the testing and evaluation of the medical materials in terms of acute systemic toxicity by using in vivo experimental animal model. The acute systemic toxicity test gives information about the potential hazardous effects of the medical device, material and/or their extracts in a recommended animal model in single or multiple exposure for a period of less than 24 hours.

Reason and Method of Test Application

5 ea. male CD1 mice were used for the spinal implant sample as the test group and also 5 ea. male CD1 mice were used as control.

In accordance with the protocol "ISO 10993-12 Biological evaluation of medical devices -- Part 12: Sample Preparation", as the sample shows a solid and shapeless structure and it cannot be directly applied to the experimental animal, the extract preparation method was preferred. According to the title 10.3.1 of the relevant protocol, incubation was applied for 72 hours at 37°C and, according to the Table 1 given under the title 10.3.3, the extraction preparation rate, however, was determined as 0,2 g sample/ml.

Test Animal

Species	Mouse	
Race	CD1	
Gender	Male	
Number	Test	5
	Control	5
Age	8-10 weeks	
Shelter condition	Individual conventional euro type-1 cage set	

Remarks:

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Initial Weights (gr)

Test 1	22,5	**Kontrol 1**	21,6
Test 2	26,5	**Kontrol 2**	20,4
Test 3	27,8	**Kontrol 3**	20,4
Test 4	24,3	**Kontrol 4**	24,5
Test 5	23,3	**Kontrol 5**	24,3

Administration

Administration Dose	50ml/kg	
Route of Administration	Intraperitoneal	
Ambient Conditions	Heat	22 ± 3°C
	Humidity	
	Lighting/Photoperiod	12 hours luminousness, 12 hours darkness
Nutrition	Ad-libutum commercial rodent pellet feed and automatized waterer	

Test Results

Test	**Initial Weight (gr)**	**End Weight (gr)**	**Change* Ratio**
Test 1	22,5	21	-6,67%
Test 2	26,5	26,8	1,13%
Test 3	27,8	27,4	-1,44%
Test 4	24,3	23,8	-2,06%
Test 5	23,3	25,2	8,15%
Control 1	21,6	24,4	12,96%
Control 2	20,4	22	7,84%
Control 3	20,4	23	12,75%
Control 4	24,5	25	2,04%
Control 5	24,3	26,7	9,88%

* When the weight loss is at the rate of ≥ 10%, it is considered as clinical finding.

Remarks:

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Clinical Observation and Evaluation

- The food and water consumption of all groups is normal.
- The weight changes are within the normal limits.

Its systemic effects on ISO 10993-11 and ISO 10993-12 were followed up according to the following clinical observation criteria. No negative clinical finding was encountered in any individual included in all groups.

CLINICAL OBSERVATION	OBSERVATIONS	SYSTEMS TO BE MONITORED
Respiration	Dyspnea (Abdominal respiration), apnoe, cynosis, tachypnea	Central nervous system (CNS), circulation, cardiac
Motor activities	Decreasing/increasing anesthesia, ataxia, indefinite positions, tremors	Central nervous system (CNS), somatomotor, sensory, atomic, muscle-nerve
Convulsion	Clonic, tonic, tonic-klonik asphyxial, opisthonotonos	Central nervous system (CNS), respiration, muscle-nerve, atomic
Reflexes	Corneal, righting, myotact, slight initial reflex	Central nervous system (CNS), sensory, atomic, muscle-nerve
Ocular Signs	Lacrymation, miosis, mydriasis, exophtalamos, ptosis, opacity, iritis, conjunctivitis, chromocryarhea	Atomic, irritation
Cardiovascular Signs	Bradycardia, tachycardia, arythmia, vasodilatation, vasoconstriction	Central nervous system (CNS), Atomic, cardiac, circulation
Salivation	Excessive	Atomic
Pilaoerection	Shags	Atomic
Analgesia	Reaction decrease	Central nervous system (CNS), sensory
Muscular Tonus	Hypotonia, hypertonia	Atomic
Gastrointestinal	Soft, diarhea, emesis, diuresis, rhinosis	Central nervous system (CNS), atomic, sensory, kidney, DI motility
Skin	Oedema, redness	Tissue injury, irritation

Result

During the observation period, the test and control groups were observed and evaluated by taking into account the specified criteria. According to the data, clinical symptoms, death ratios and gross pathology findings obtained with the observations made during and at the end of the test, **it was decided that** the tested product **has not acute toxicity effect.**

Remarks:

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4- Skin Sensitization Test

The sensitization test was performed by taking into account the standard protocol "ISO 10993-10 Tests for irritation and delayed-type hypersensitivity".

The sensitization tests of the samples were performed by using adult female guinea pigs (*Cavia porcellus)* at the weight of between 300 and 500 gr. As specified in the document titled ISO 10993-10, the tests were performed by administering the material to be tested subcutaneously at the amount of 0,1 ml. The topical administration, however, was applied to the left area of the animal on the 7th day and to the right area of the animal on the 14th day of the test to the area where the subcutaneous injection (intradermal induction phase) was not applied. For this, the administration plan applied onto the experimental animals is shown in the Figure 2.

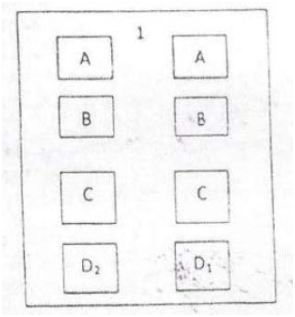

Figure 2.
1- Head part of the experimental animal.
A- Test areas into which Freund's Complete Adjuvant (FCA) and serum physiological solution were mixed at the rate of 50:50 and administered.
B- Test areas into which only the test material was administered.
C- Test areas into which the sample administered into the area A and the test material administered into the area B were mixed at the rate of 50:50 and administered.
D- The test material the topical administration of which to the intraskapular area is 0,3 ml was administered. In the administrations made into the areas A, B and C, one pair of 0,1 ml injection was made to the right and left areas of each animal.
In the area D, however, the topical administration was made to the right area (D_1) on the 7th day and to the left area (D_2) on the 14th day.

Remarks:		
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Negative Control
The negative control was performed comparatively in 2 different administrations in 2 different areas (Figure 3).

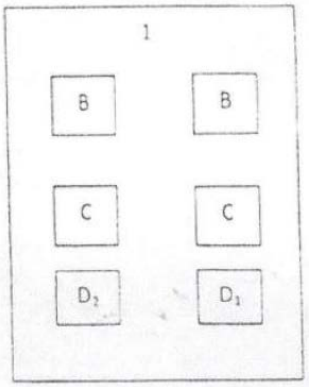

Figure 3.
1- Head part of the experimental animal.
B- Serum Physiological 0,1 ml.
C- (FCA) and serum physiological solution were administered by mixing at the rate of 50:50.
D- 0,3 ml serum physiological was administered to the topical areas.

One day after the experimental animals were shaved in order to provide the sufficient administration area, the test materials were administered as shown in the Figure 2 and in the control animals as shown in the Figure 3. All administrations were made so as to be 0,1 ml subcutaneously. After the administration, the areas were not closed in any way. In the topical administration, however, the test material was administered in the experimental animals and 0,3 ml serum physiological was administered onto the skin in the control animals. After the administration, the area was closed with a steril gauze dressing and all administration areas were wrapped with elastic bandage. It was ensured that the gauze dressings contacted with the area for 48 hours. At the end of the administration period, the bandages were opened and the reactions which occurred on the skin were noted down. The second topical administration was made after 7 days and the same experimental procedures were followed.

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Administered test materials
In administration, 10 animals were used for the test material and 5 animals were used for the control.

Test Materail: Spinal Implant

Reaction	Classification Scale
There is no visible change	0
Marked or patchy rubescence	1
Medium or confluent rubescence	2
Intense rubescence and blistering	3

Table 6. Evaluation criterion and scoring.

Evaluation Average

Samples	Result
Spinal Implant	0,9
Control	0,3

Table 7. Average score values.

Result

Observations and evaluations were made during the administered experimental phases as specified in the protocol and the administration areas for each observation were scored by taking the evaluation scoring criteria in Table 6. into consideration. Very rare mild rubescence with patches were found on the skin. The score value for the "spinal implant" product, which was tested by taking the average of the scores obtained as soon as the test period was completed, was set at 0,9. **As a result,** it has been determined that the product tested according to the protocol evaluation criteria specified in the ISO 10993-10 document **does not have the sensitive (substance sensitive) characteristic** according to the obtained findings and score values.

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5- Cytotoxicity Test
Cytotoxicity tests were performed by taking the "Biological assessment of medical products, the ISO 10993-5 in vitro cytotoxicity test" standards into consideration.

Test start date: 20.02.2012
Test ending date: 23.02.2012

Completion of the sample:
Sample features are as described in section 1. Examples were supplied by the "NORMMED MED. VE MAK. SAN. TİC. LTD. ŞTİ." company.

Description of the used cell strain and cause:
L929 mouse cell straine was used. It was selected for being one of the cell straines recommended by ISO 10993-5 and for its suitability to represent the mammalian system.

The name and the party number of the company, where the used feedlot was supplied, added serum and antibiotics:
As a feedlot, DMEM / F12 (Gibro Cat # 32500-035, lot # 614238) + 10% Fetal bovine serum (Biochrom AG Cat # S0115, lot # 0827H) + penicillin streptomycin (Biological Industries, Cat # 03- 031-1C, lot # 655265) in) were used.

Test method and rational:
- Method: Extraction method
Rational: To analyze the toxic effect of dissolvable materials.
- Cytotoxicity Measurement Method: WST- 1 cell viability analysis (Colorimetric)
Rational: To be able to measure cell viability consistently and sensitively

Remarks:

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Extraction protocol (nature and concentration of the leachable material if appropriate):
The samples were randomly selected in accordance with the principles set out in ISO 1099-12, and the selected vertebrate implants (10 x 4 x 3 mm) were > 1,0 mm, so that the extraction rate was 3 cm^2 / ml (surface area cm^2/ extract solution, ml), were incubated (37 ° C) by being shaken for 72 hours at 120 rpm inside the feedlot and the extracts were prepared.

Experimenl protocol:
One day before the experiment, L929 cells were counted and cultured in wells of 96 cels to be 1 x 10^4 cells / well. Ready-72 hour extracts and controls were added to the cells without any incubation and incubation was carried out for 24 hours at 37 ° C, 5% CO_2, followed by viability determinations with WST-1 agent.

Negative, positive and other controls:
Negative controls:
Control # 1: Fresh feedlot containing serum without any administered procedure, DMEM- F12 fresh.
Control # 2: Feedlot not containing any sample, containin serum that is incubated in the same conditions as the samples DME-F 12 extract
Control # 3: Negative control, RAUMEDIC Tubes in silicone rubber grade RAUMEDIC- SIK 8363

Positive controls:
Control #1: Positive control, RAUMEDIC PVC Orc. Sn.

Remarks:

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Cell response and other observations:
Quantitative Evaluation of Extraction Method:

	Spinal Implant		**Controls**
Spine Implant Extract (72 hours)	0	DMEM- F12 fresh	0
		DMEM- F12 Ekstrakt	0
		Negatif control	0
		Positive control	4

Degree	Reactivity	Status
0	None	Intracytoplasmic granules are prominent, no cell lysis, no effect on cell growth
1	Low	Became rounded, no weak-binding and/or intracytoplasmic granules, morphologically altered, the presence of lysed cells in between is less than 20%
2	Mild	Became rounded, the ratio of the cells with no intracytoplasmic granules is below 50%, no extensive cell lysis, growth inhibition below 50%
3	Medium	Less than 70% of the cells are rounded or lysed and growth inhibition is not more than 50%.
4	Serious	Nearly all or all of the cells are tosted.

Table 8. Evaluation criteria and scoring for cytotoxicity testing.

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Result

According to the evaluation criteria given in the Table 8, the potential of "Spinal Extract" for causing cell death as compared with the control on the L929 cell culture at the end of its 72-hour incubation was examined and it was observed that the tested product is not **cytotoxic** according to the result of ISO 10993-5 cytotoxicity test performed within the scope of the biocompatibility.

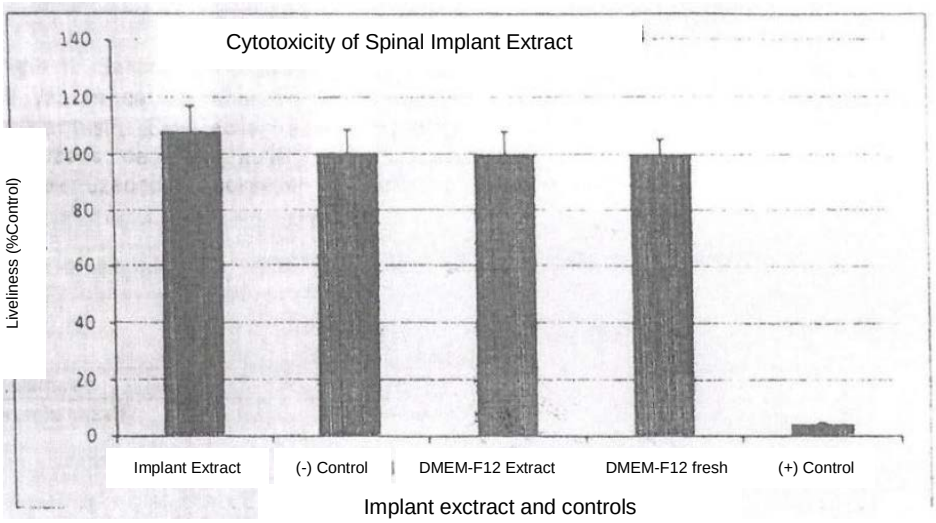

Figure 1. In the Figure, the liveliness analyses performed with the extracts prepared from the sample called as "Spinal Implant are shown. The negative and positive controls are clarified above. The absorbance values obtained were normalized by using the absorbance values of the negative control 3 for 100% liveliness. For instance, it was observed that it has no cytotoxic effect on the cells as compared with the controls.

The data obtained for each sample was obtained as a result of triplike study of 3 extracts belonging to 3 randomly selected from one sample in the tests.

Remarks:

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ANALYSIS REPORT

(Industrial Technical Support Service)

Report no : B.14.2.TBT.5.01.13.00-181.06.03.126/**3451 (ADDITIONAL REPORT 2)**

Report date : **10.06.2014**

Requested by : NORMMED MED. VE MAK. SAN. TIC. LTD. STI.

Address : IVEDIK ORG. SAN. AGAC ISLERI SAN. SIT. 521. SOK. NO: 43 YENIMAHALLE, ANKARA

Subject : INTRADERMAL IRRITATION AND GENOTOXICITY TEST PERFORMED FOR THE "SILVER COATED SPINAL SCREEW" PRODUCT WITHIN THE SCOPE OF BIOCOMPATIBILITY TESTS

The results contained in this report belongs to the examined sample only.

Approved by

// Official signature //

Assoc. Prof. Dr. Fatima YUCEL
GMBE Industrial Services Supervisor

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Requested by	: NORMMED MED. VE MAK. SAN. TIC. LTD. STI.		
Address	: IVEDIK ORG. SAN. AGAC ISLERI SAN. SIT. 521. SOK. NO: 43 YENIMAHALLE, ANKARA		
Sample	: Single type sample	**Expiration date**	: -
Number of samples	: 14 ea.	**Production Date**	: -
Supply manner of the sample	: Cargo	**Institute sample record no**	: 12/44-GMBE
Condition of the sample at the time of acceptance	: They were supplied under nonsterile conditions in an envelope.	**Date and time of acceptance**	: 21.04.2014
		Date of analysis	: 28.04.2014-09.06.2014

Witness sample info:	( ) Return to the customer	(x) Witness sample is available	( ) Witness sample was not taken.

1- Samples

The irritation and genotoxicity test were performed on 14 ea. samples called "silver coated spinal screw" upon the application of Normmed Medikal ve Makine San. Tic. Ltd. Sti.

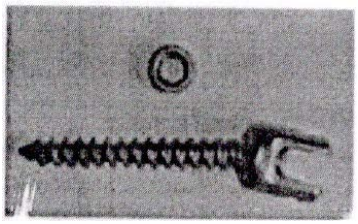
Sample	Characteristic	Item
Silver Coated Spinal Screw	The product is at the diameter of 6,5 mm and at the length of 45 mm. It is used as spinal implant.  **Figure 1.** Photograph of the product.	14

Table 1: Tested sample.

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2- Intradermal Irritation Test:
The intradermal irritation test was performed as clarified in the "Annex B" part of the international protocol "ISO 10993-10:2010 Tests for irritation and delayed-type hypersensitivity". Apart from this, the standards "ISO 10993-2:2006 Animal Welfare Requirements" and "ISO 10993-12:2012 Sample Preparation and Reference Materials" were also taken into account.

2.1. Experimental Animals used and Shelter Conditions
As recommended in the standard protocol, 8-12-week (the difference between their weights is less than ±20%) 3 ea. female New Zeland rabbits at the weight of not less than 2 kg were used as the experimental animals. The rabbits used in the test were randomly selected, marked one by one and taken into separate cages 24 hours before the administration. Whether there is any deformation in the experimental animals and their general health status were controlled before the administration. The shelter conditions were adjusted in such a manner that the room temperature would be 22°C (± 3°C), the humidity would be between 30% and 70% and so as to be 12-hour luminousness and 12-hour darkness. The rabbits used in the test were kept in separate cages during the test.

2.2. Preparation of the Samples
As stated in the document titled ISO 10993-10:2010, the extraction protocol recommended for the samples which will not be directly administered was applied. For this, 3 cm^2/ml surface area/volume ratio was administered and subjected to 72-hour incubation at 37°C in compliance with the form and structure of the product. PBS (phosphate buffer saline) was used as polar solvent and corn oil was used as non-polar solvent.

2.3. Administration Protocol
After the experimental animals were shaved enough to provide sufficient administration area, the samples were administered by intradermal injection as shown in the Figure 1. 0,2 ml substance in total was administered to 5 different points in each test area. Also as seen in the Figure 1, polar extract was administered to the area no 2, non-polar extract to the area no 4, polar solvent control to the area no 3 and non-polar solvent control to the area no 5. The around the injection areas was defined by marking. After the administration, each injection area was observed at the 24th, 48th and 72nd hours and scored by evaluating according to the criteria set forth in the Table 2.

2.4. Evaluation of the Results
While calculating *the score value* for the tested samples and controls, the redness and oedema values obtained for each observation time are added and divided by 15 (3 observation times X 5 observation areas). For the tested samples and controls, *the general average score* is obtained by dividing the obtained score value by the number of the animals, i.e. 3. *The final test sample score*, however, is obtained by the substraction of the value obtained for blank, if any, from the score obtained from the test sample. If the final test sample score obtained for the test samples is 1 or lower than 1, it is deemed that the requirements of the test have been fulfilled.

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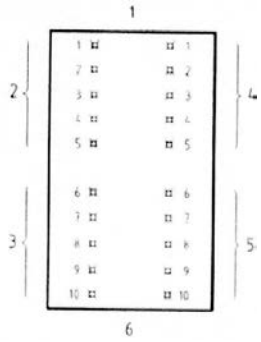

Figure 1. 1; Head part of the experimental animal, 2; polar extract test area, 3; polar solvent control area, 4; non-polar extract test area, 5; non-polar solvent control area, 6; tail part of the experimental animal.

Reaction	Score
Redness and scar formation	
No redness	0
Very slight redness (hardly noticable)	1
Visibly redness	2
Medium redness	3
Serious redness and scar formation	4
Oedema	
No oedema	0
Very slight oedema (hardly noticable)	1
Visibly oedema	2
Medium oedema (approximately 1 mm)	3
Serious oedema (larger than 1 mm)	4
Total possible score for irritation	8
Other undesired changes that might be observed on the skin should be also recorded and reported.	

Table 2. Evaluation criteria and scoring.

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2.5. Observation Results

All observation scores and the general average scores obtained were calculated as described in the Part 2.4 and are submitted in the Tables 3 and 4, respectively.

Experimental Animal ID	Samples	Area	Observation (Hour)					
			Redness			Oedema		
			24	48	72	24	48	72
1	*Silver coated spinal screw (polar)*	*Left Front Area*	0	0	0	0	0	0
	Silver coated spinal screw (non-polar)	*Right Front Area*	0	0	0	0	0	0
	Polar Solvent Control	*Left Rear Area*	0	0	0	0	0	0
	Non-polar Solvent Control	*Right Rear Area*	0	0	0	0	0	0
2	*Silver coated spinal screw (polar)*	*Left Front Area*	0	0	0	0	0	0
	Silver coated spinal screw (non-polar)	*Right Front Area*	0	0	0	0	0	0
	Polar Solvent Control	*Left Rear Area*	0	0	0	0	0	0
	Non-polar Solvent Control	*Right Rear Area*	0	0	0	0	0	0
3	*Silver coated spinal screw (polar)*	*Left Front Area*	0	0	0	0	0	0
	Silver coated spinal screw (non-polar)	*Right Front Area*	0	0	0	0	0	0
	Polar Solvent Control	*Left Rear Area*	0	0	0	0	0	0
	Non-polar Solvent Control	*Right Rear Area*	0	0	0	0	0	0

Table 3. Evaluation results for the silver coated spinal screw.

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http://www.mam.gov.tr

Report no	: B.14.2.TBT.5.01.13.00-181.06.03.126/**3451 (ADDITIONAL REPORT 2)**

Samples	**Area**	**General Average Score**
Silver coated spinal screw (polar)	*Left Front Area*	0
Silver coated spinal screw (non-polar)	*Right Front Area*	0
Polar Solvent Control	*Left Rear Area*	0
Non-polar Solvent Control	*Right Rear Area*	0

Table 4. General average score values.

2.5. Result

For the test material, as stated, the general average score was obtained (Table 4) by averaging the values obtained after three different observations were made (Table 3) for two different criteria. In the observations made for the tested samples, no oedema or redness was observed. In line with the results obtained, **it was found that** the product defined as "silver coated spinal screw" tested **has not intradermal irritating characteristic** according to the protocol and evaluation criteria specified in the document ISO 10993-10: 2010.

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http://www.mam.gov.tr

Report no	: B.14.2.TBT.5.01.13.00-181.06.03.126/**3451 (ADDITIONAL REPORT 2)**

3- Bacterial Reverse Mutation Test - AMES Test

The AMES test was performed by using the test materials and Salmonella strains supplied from Molecular Toxicology (Moltox) Company in accordance with "OECD Guideline for Testing of Chemicals: Bacterial Reverse Mutation Test (No: 471, Adopted: 21st July 1997)". The genotype information of *S. typhimurium* strains used in the analysis is given in the Tabel 5.

Strain•	Affected Gene	DNA repair	Lipopolisaccaride	Plazmids	Mutation Type
S. typhimurium TA1535	*hisG46*	*uvrB*	*rfa*	Does not contain plasmid.	Base-pair change
S. typhimurium TA97a	*hisD6610* *hisO1242*	*uvrB*	*rfa*	pKM101	Frameshift
S. typhimurium TA98	*hisD3052*	*uvrB*	*rfa*	pKM101	Frameshift
S. typhimurium TA100	*hisG46*	*uvrB*	*rfa*	pKM101	Base-pair change
S. typhimurium TA102	*hisG428*	-	*rfa*	pKM101	Base-pair change

Table 5. Genotype information of *S. typhimurium* strains.

*These strains need the presence of histidine which is an essential amino acid for growing. The trace amount of histidine necessary in the analysis is provided by being added to top agar. When the reverse mutation occurs, the occurrence of reverse mutant colony in the minimal bacterium nutrient medias containing glucose is observed.

Preparation of *S. typhimurium* Cultures

The *S. typhimurium* cultures that were used in the test were prepared as follows:

- *S. typhimurium* TA1535 disc was thrown into 20 ml Oxoid#2 Nutrient Broth,
- Each of *S. typhimurium* TA97a, TA98 and TA100 discs was thrown into 20 ml Oxoid#2 Nutrient Broth containing ampicilline antibiotic in such a manner that the last concentration would be 25 µg/ml.
- *S. typhimurium* TA102 disc was thrown into 20 ml Oxoid#2 Nutrient Broth containing ampicilline and tetracycline antibiotics in such a manner that the last concentrations would be 25 µg/ml and 2 µg/ml, respectively.

The cultures initiated were left to reproduction at 100 rpm at 37°C during the night (15-16 hours). After the incubation, the absorbance of each culture in 660 nm was determined and the test was started when the absorbance was within the range of 1.0-1.2. During the test, the cultures were kept at the room temperature for them not to be affected by thermal shock and the culture flasks were closed with aluminium folio in order to protect them from light. In the test, 100 µl culture was used for each plate.

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Phenotypic Control of the Strains
The strains that were used were controlled in four-partition phenotype verification plate phenotypically. The observations expected in different partitions of this plate are as in the Table 6.

Partition	Expected Observation	Genotype
1	No growing is observed in any strain.	*his-*
2	For all strains, zonal inhibition is observed around the rifampicin-impegnated crystal viole (CV) disc.	*rfa*
3	Growing is observed in all strains except for *S. typhimurium* TA 1535 strain.	pKM101
4	Only TA102 strain grows.	pAQ1 (bears tetracyline resistance gene)

Table 6. Observations expected in the phenotype verification plate.

In the analysis, a phenotype verification plate was used for each strain. A drawing from the culture of the relevant strain was made to each partition in the plate. In each plate, rifampicin disc (CV disc) was left to the partition no 2. The plates were incubated for 24-48 hours at 37°C.

Positive Control
Sodium Azide [CAS no. 26628-22-8] was used as positive control for *S. typhimurium* TA1535 and TA100 strains, **ICR 191 Acridine** [CAS no. 17070-45-02] for TA97a strain, **Daunomycin** [CAS no. 23541-50-6] for TA98, **Mitomycin C** [CAS no. 50-07-7] for TA102 strain. In order to perform the metabolic activation control, however, **2-Aminoanthracene** [CAS no. 613-13-8] was used as positive control for all *S. typhimurium* strains tested in the presence of S9 mixture and **Benzo(a)pyrene** [CAS no. 50-32-8] for TA98 and TA100 strains.

The doses and metabolic activation information of the positive controls used in the test are given in the Table 7.

P.K. 21, 41470 GEBZE - KOCAELI
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Positive Control	*S. typhimurium* strain	Dose (µg/plate)	Metabolic Activation
Sodium Azide	TA1535 and TA100	1,5 µg	-S9
ICR 91 Acridine	TA97a	1,0 µg	-S9
Mitomycin C	TA102	0,5 µg	-S9
Daunomycin	TA98	6,0 µg	-S9
2-Aminoanthracene (activation control)	All strains	10,0 µg	+S9
Benzo(a)pyrene (activation control)	TA98 and TA100	20,0 µg	+S9

Table 7. Positive controls.

Negative Control

As negative control, phosphate buffered saline (PBS) was used in the preparation of the sample extract.

Metabolic Activation System

In the test, as the metabolic activation system, post-mitochondrial S9 fraction which was prepared from liver of the male Sprague Dawley rat induced with Aroclor 1254 supported with NADPH Regensys^{TM} a (0.1 m phosphate buffer, glucose-6-phosphate in pH 7.4, contains $MgCI_2$/KCI) and NADPH Regensys^{TM} B (NADPH) co-factors. The analysis was performed by using 10% (v/v) S9 mixture and 500 µL S9 mixture was used per plate.

Sample

The sample is "silver coated spinal screw" belonging to Normmed Medikal ve Makine Sanayi Ticaret Limited Şirketi and the characteristics of the sample are given in the Part 1. The extraction data was obtained with 75-hour incubation at 37°C by applying 3 cm^2/ml surface area/volume ratio in consideration of the form and structure of the sample in accordance with ISO10993-12: 2012 Sample Preparation and Reference Materials Standard Protocol". PBS was used for the extraction. The sample extract was taken into the test without being waited and 100 µl extract was used per plate.

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http://www.mam.gov.tr

Report no	: B.14.2.TBT.5.01.13.00-181.06.03.126/**3451 (ADDITIONAL REPORT 2)**

Other Information regarding the Analysis

The bacterial reverse mutation test was performed according to "the standard plate incorporation method" in the presence and absence of the metabolic activation system in order to evaluate the mutagenicity of the sample in *S. typhimurium* TA1635, TA97a, TA98, TA100 and TA102 strains. For each strain, S9 (+/-) samples of the negative (PBS) control and S9 (-) sample of the positive (mutagen) control were prepared. If it was a mutagen exposed to metabolic activation, S9 (+) sample of the positive control and S9 (+/-) samples of the sample were prepared. After the samples were mixed with top agar containing histidine/biotin, they were poured into the minimal glucose agar plates. All plates that were used in the analysis were left to incubation for 48-72 hours in an incubator at 37°C. The analysis was performed as two repetitive analysis for all samples.The revertant (reverse mutant) colonies which occurred in each plate after the incubation were manually counted. The average colony number for each duplicated study was determined. The numbers obtained for the reverse mutant colonies of each strain in the sample and controls are submitted in the form of a table in the test findings section.

Test Findings

TA1535 strain	S9	Number of Reverse Mutant Colony Plate #1	Number of Reverse Mutant Colony Plate #2	Average Number of Reverse Mutant Colony	Standard Deviation (±)
Sample	+	12	14	13	1,41
Sample	-	10	14	12	2,83
2-Aminoanthracene	+	164	135	149	20,51
2-Aminoanthracene	-	25	24	24	0,71
Sodium Azide	-	495	604	549	77,07
Negative control (PBS)	+	10	12	11	1,41
Negative control (PBS)	-	12	20	16	5,66

Table 8. Results of TA1535 strain.

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http://www.mam.gov.tr

Report no	: B.14.2.TBT.5.01.13.00-181.06.03.126/**3451 (ADDITIONAL REPORT 2)**

TA97a strain	S9	Number of Reverse Mutant Colony Plate #1	Number of Reverse Mutant Colony Plate #2	Average Number of Reverse Mutant Colony	Standard Deviation (±)
Sample	+	57	63	60	4,24
Sample	-	67	50	58	12,02
2-Aminoanthracene	+	354	468	411	80,61
2-Aminoanthracene	-	134	139	136	3,54
ICR 191 Acridine	-	481	206	343	194,45
Negative control (PBS)	+	48	107	77	41,72
Negative control (PBS)	-	50	85	67	24,75

Table 9. Results of TA97a strain.

TA98 strain	S9	Number of Reverse Mutant Colony Plate #1	Number of Reverse Mutant Colony Plate #2	Average Number of Reverse Mutant Colony	Standard Deviation (±)
Sample	+	29	40	34	7,78
Sample	-	20	23	21	2,12
2-Aminoanthracene	+	1650	1672	1661	15,56
2-Aminoanthracene	-	77	93	85	11,31
Benzo(a)pyrene	+	594	565	579	20,51
Benzo(a)pyrene	-	29	23	26	4,24
Daunomycin	-	1016	1045	1030	20,51
Negative control (PBS)	+	33	32	32	0,71
Negative control (PBS)	-	29	23	26	4,24

Table 10. Results of TA98 strain.

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Report no	: B.14.2.TBT.5.01.13.00-181.06.03.126/**3451 (ADDITIONAL REPORT 2)**

TA100 strain	S9	Number of Reverse Mutant Colony Plate #1	Number of Reverse Mutant Colony Plate #2	Average Number of Reverse Mutant Colony	Standard Deviation (±)
Sample	+	132	148	140	11,31
Sample	-	78	94	86	11,31
2-Aminoanthracene	+	2286	2310	2298	16,97
2-Aminoanthracene	-	114	107	110	4,95
Benzo(a)pyrene	+	787	845	816	41,01
Benzo(a)pyrene	-	95	82	88	9,19
Sodium Azide	-	597	604	600	4,95
Negative control (PBS)	+	111	129	120	12,73
Negative control (PBS)	-	91	80	85	7,78

Table 11. Results of TA100 strain.

TA102 strain	S9	Number of Reverse Mutant Colony Plate #1	Number of Reverse Mutant Colony Plate #2	Average Number of Reverse Mutant Colony	Standard Deviation (±)
Sample	+	337	376	356	27,58
Sample	-	357	369	363	8,49
2-Aminoanthracene	+	766	785	775	13,44
2-Aminoanthracene	-	597	531	564	46,67
Mitomycin C	-	795	812	803	12,02
Negative control (PBS)	+	351	318	334	23,33
Negative control (PBS)	-	336	362	349	18,38

Table 12. Results of TA102 strain.

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Evaluation of the Test Findings and Result

After the incubation, the phenotypes compatible with the genotypes of *S. typhimurium* strains that were used in the test in the phenotype verification plates were obtained. The His* reverse mutants growing in the minimal glucose agar plates were easily counted. When the findings obtained with five *S. typhimurium* strains were considered, the AMES test of the sample was considered negative (-) because fold increase equal to or above 2 was not observed in the number of His* reverse mutant colonies of the sample as compared with the negative control. **It was determined that the product defined as "silver coated spinal screw" does not generate mutation (is not mutagenic) under the test conditions and in the bacterium strains used.**

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