giving a continuous record of the blood pressure. Insert into the femoral vein another cannula, filled with a heparinised 9 g/l solution of sodium chloride, through which can be injected the solutions of histamine and of the substance to be examined. Determine the sensitivity of the animal to histamine by injecting intravenously at regular intervals, doses of *histamine solution R* corresponding to 0.1 µg and 0.15 µg of histamine base per kilogram of body mass. Repeat the lower dose at least 3 times. Administer the second and subsequent injections not less than 1 min after the blood pressure has returned to the level it was at immediately before the previous injection. The animal is used for the test only if a readily discernible decrease in blood pressure that is constant for the lower dose is obtained and if the higher dose causes greater responses. Dissolve the substance to be examined in sufficient of a 9 g/l solution of sodium chloride or other prescribed solvent, to give the prescribed concentration. Inject intravenously per kilogram of body mass 1.0 ml of *histamine solution R*, followed by 2 successive injections of the prescribed amount of the solution to be examined and, finally, 1.0 ml of *histamine solution R*. The second, third and fourth injections are given not less than 1 min after the blood pressure has returned to the level it was at immediately before the preceding injection. Repeat this series of injections twice and conclude the test by giving 1.5 ml of *histamine solution R* per kilogram of body mass.

If the response to 1.5 ml of *histamine solution R* per kilogram of body mass is not greater than that to 1.0 ml the test is invalid. The substance to be examined fails the test if the mean of the series of responses to the substance is greater than the mean of the responses to 1.0 ml of *histamine solution R* per kilogram of body mass or if any one dose of the substance causes a greater depressor response than the concluding dose of the histamine solution. The test animal must not be used in another test for depressor substances if the second criterion applies or if the response to the high dose of histamine given after the administration of the substance to be examined is less than the mean response to the low doses of histamine previously injected.

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2.6.12. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS (TOTAL VIABLE AEROBIC COUNT)

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi which may grow under aerobic conditions.

The tests are designed primarily to determine whether or not a substance that is the subject of a monograph in the Pharmacopoeia complies with the microbiological requirements specified in the monograph in question. When used for such purposes follow the instructions given below, including the number of samples to be taken and interpret the results as stated below. The tests may also be used for the test for *Efficacy of antimicrobial preservation* (*5.1.3*) as described in the Pharmacopoeia. They may furthermore be used for monitoring raw material quality and may be used in association with guidelines on *Microbiological quality of pharmaceutical preparations* (*5.1.4*). When used for such purposes, for example by a manufacturer for raw materials and/or finished product monitoring or for process validation, the conduct of the tests including the number of samples to be taken and the interpretation of the results are matters for agreement between the manufacturer and the competent authority.

Carry out the determination under conditions designed to avoid accidental contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any micro-organisms which are revealed in the test. If the product to be examined has antimicrobial activity this must be adequately neutralised. If inactivators are used for this purpose their efficacy and non-toxicity versus micro-organisms are demonstrated.

Determine the total viable aerobic count by the membrane filtration method, or the plate-count method as prescribed in the monograph.

The Most Probable Number (MPN) method is reserved for bacterial counts when no other method is available. The choice of a method may be based on factors such as the nature of the product and the expected number of micro-organisms. Any method which is chosen must be properly validated.

When used in conjunction with chapter *5.1.3* or *5.1.4*, the pour-plate method, the surface-spread method and the membrane filtration method may be used.

PREPARATION OF THE SAMPLE

Sampling plan. Sampling of the product must follow a well-defined sampling plan. The sampling plan will be dependent on factors such as batch size, health hazard associated with unacceptably highly contaminated products, the characteristics of the product and the expected level of contamination. Unless otherwise prescribed, use sample(s) of 10 g or 10 ml of the substance or preparation to be examined taken with the precautions referred to above. Select the sample(s) at random from the bulk material or from the available containers of the preparation. If necessary, to obtain the required quantity, mix the contents of a sufficient number of containers to provide each sample, depending on the nature of the substance or preparation to be examined.

An example of a sampling plan applicable to products where homogeneity with respect to the distribution of micro-organisms may be a problem, is the three-class sampling plan. In this case five samples from each batch are drawn and investigated separately. The three recognised classes are:

(i) acceptable samples, i.e. samples containing less than m CFU (colony-forming units) per gram or millilitre, where m is the limit specified in the relevant monograph;

(ii) marginal samples, i.e. with more than m CFU, but less than $10m$ CFU per gram or millilitre;

(iii) defective samples, i.e. containing more than $10m$ CFU per gram or millilitre.

Water-soluble products. Dissolve or dilute 10 g or 10 ml of the product to be examined in buffered sodium chloride-peptone solution pH 7.0 or in another suitable liquid. In general a one in ten dilution is prepared. However, the characteristics of the product, or the required sensitivity may necessitate the use of other ratios. If the product is known to have antimicrobial activity, an inactivating agent may be added to the diluent. If necessary adjust the pH to about pH 7 and prepare further serial tenfold dilutions using the same diluent.

Non-fatty products insoluble in water. Suspend 10 g or 10 ml of the product to be examined in buffered sodium chloride-peptone solution pH 7.0 or in another suitable liquid. In general a one in ten suspension is prepared, but the characteristics of some products may necessitate the use of larger volumes. A suitable surface-active agent such as 1 g/l of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If the product is known to have antimicrobial activity, an inactivating agent

may be added to the diluent. If necessary adjust the pH to about pH 7 and prepare further serial tenfold dilutions using the same diluent.

Fatty products. Homogenise 10 g or 10 ml of the product to be examined with not more than half its weight of sterile polysorbate 80 or another suitable sterile surface-active agent, heated if necessary to not more than 40 °C, in exceptional cases to not more than 45 °C. Mix carefully and if necessary maintain the temperature in a water-bath or in an incubator. Add sufficient pre-warmed buffered sodium chloride-peptone solution pH 7.0 to make a one in ten dilution of the original product. Mix carefully whilst maintaining the temperature for the shortest time necessary for the formation of an emulsion and in any case for not more than 30 min. Further serial tenfold dilutions may be prepared using buffered sodium chloride-peptone solution pH 7.0 containing a suitable concentration of sterile polysorbate 80 or another sterile surface-active agent.

Transdermal patches. Remove the protective cover sheets ("release liner") of ten patches of the transdermal preparation by using sterile forceps and place them, the adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with sterile gauze (or woven-filter type monofilament polymer grid), if necessary, and transfer the ten patches to a minimum volume of 500 ml of buffered sodium chloride-peptone solution pH 7.0 containing suitable inactivators such as polysorbate 80 and/or lecithin. Shake vigorously the preparation for at least 30 min (preparation A). Prepare another ten patches in the same way, place them in a minimum volume of 500 ml of broth medium D and shake vigorously for at least 30 min (preparation B).

EXAMINATION OF THE SAMPLE

Membrane filtration. Use membrane filters having a nominal pore size not greater than 0.45 µm and whose effectiveness to retain bacteria has been established. The type of filter material is chosen in such a way that the bacteria retaining efficiency is not affected by the components of the sample to be investigated. Cellulose nitrate filters, for example, may be used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters, for example, for strongly alcoholic solutions. The filtration apparatus is designed to allow the transfer of the filter to the culture medium.

Transfer a suitable amount of the sample prepared as described in the section Preparation of the sample (preferably representing 1 g of the product, or less if large numbers of colony-forming units are expected) to each of two membrane filters and filter immediately. Wash each filter with three quantities, each of about 100 ml of a suitable liquid such as buffered sodium chloride-peptone solution pH 7.0. To this solution, surface-active agents such as polysorbate 80, or inactivators of antimicrobial agents may be added. If validated, less than three washes may be applied. Transfer one of the membrane filters, intended primarily for the enumeration of bacteria, to the surface of a suitable agar medium, such as medium B and the other, intended primarily for the enumeration of fungi, to the surface of a suitable agar medium, such as medium C. Incubate the plate of agar medium B at 30 °C to 35 °C, and the plate of agar medium C at 20 °C to 25 °C for five days, unless a reliable count is obtained in a shorter time. Select plates with the highest number less than 100 colonies and calculate the number of colony-forming units per gram or millilitre of product.

When examining transdermal patches, filter 50 ml of preparation A separately through each of two sterile filter membranes. Place one membrane to agar medium B for total aerobic microbial count, the other membrane to agar medium C for the count of fungi.

PLATE-COUNT METHODS

a. Pour-plate method. Using Petri dishes 9 cm in diameter, add to each dish 1 ml of the sample prepared as described in the section Preparation of the sample and 15 ml to 20 ml of a liquefied agar medium suitable for the cultivation of bacteria (such as medium B), or 15 ml to 20 ml of a liquefied agar medium suitable for the cultivation of fungi (such as medium C) at not more than 45 °C. If larger Petri dishes are used the amount of agar is increased accordingly. Prepare for each medium at least two Petri dishes for each level of dilution. Incubate the plates at 30 °C to 35 °C (20 °C to 25 °C for fungi) for five days, unless a reliable count is obtained in a shorter time. Select the plates corresponding to one dilution and showing the highest number of colonies less than 300 (100 colonies for fungi). Take the arithmetic average of the counts and calculate the number of colony-forming units per gram or millilitre.

b. Surface-spread method. Using Petri dishes 9 cm in diameter, add 15 ml to 20 ml of a liquefied agar medium suitable for the cultivation of bacteria (such as medium B) or a liquefied agar medium suitable for the cultivation of fungi (such as medium C) at about 45 °C to each Petri dish and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example in a LAF bench or in an incubator. Spread a measured volume of not less than 0.1 ml of the sample prepared as described in the section Preparation of the sample over the surface of the medium. Use at least two Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of colony-forming units proceed as described for the pour-plate method.

MOST-PROBABLE-NUMBER METHOD

The precision and accuracy of the most-probable-number method (MPN) is less than that of the membrane filtration method or the plate-count methods. Unreliable results are obtained particularly for the enumeration of moulds. For these reasons the MPN method is reserved for the enumeration of bacteria in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least three subsequent tenfold dilutions of the product as described in the section Preparation of the sample. From each level of dilution three aliquots of 1 g or 1 ml are used to inoculate three tubes with 9 ml to 10 ml of a suitable liquid medium (such as broth medium A). If necessary a surface-active agent such as polysorbate 80, or an inactivator of antimicrobial agents may be added to the medium. Thus, if three levels of dilution are prepared nine tubes are inoculated. Incubate all tubes for five days at 30 °C to 35 °C. Record for each level of dilution the number of tubes showing microbial growth. If the reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth, or on a suitable agar medium (such as agar medium B), for 18 h to 24 h at the same temperature and use these results. Determine the most probable number of bacteria per gram or millilitre of the product to be examined from Table 2.6.12.-1.

Table 2.6.12.-1. – *Most-probable-number values of bacteria*

Three tubes at each level of dilution							
Number of positive tubes			MPN per gram	Category*		95 per cent confidence limits	
0.1 g	0.01 g	0.001 g		1	2		
0	0	0	< 3			–	–
0	1	0	3		x	< 1	17
1	0	0	3	x		1	21
1	0	1	7		x	2	27
1	1	0	7	x		2	28
1	2	0	11		x	4	35
2	0	0	9	x		2	38
2	0	1	14		x	5	48
2	1	0	15	x		5	50
2	1	1	20		x	8	61
2	2	0	21	x		8	63
3	0	0	23	x		7	129
3	0	1	38	x		10	180
3	1	0	43	x		20	210
3	1	1	75	x		20	280
3	2	0	93	x		30	390
3	2	1	150	x		50	510
3	2	2	210		x	80	640
3	3	0	240	x		100	1400
3	3	1	460	x		200	2400
3	3	2	1100	x		300	4800
3	3	3	> 1100			–	–

Category 1: Normal results, obtained in 95 per cent of the cases.

Category 2: Less likely results, obtained in only 4 per cent of cases. These are not to be used for important decisions. Results that are even less likely than those of category 2 are not mentioned and are always unacceptable.

EFFECTIVENESS OF CULTURE MEDIA AND VALIDITY OF THE COUNTING METHOD

Grow the bacterial test strains separately in containers containing a suitable liquid medium (such as broth medium A) at 30 °C to 35 °C for 18 h to 24 h. Grow the fungal test strains separately on a suitable agar medium (such as medium C without antibiotics) at 20 °C to 25 °C for 48 h for *Candida albicans* and at 20 °C to 25 °C for 7 days for *Aspergillus niger*.

Staphylococcus aureus	such as ATCC 6538 (NCIMB 9518, CIP 4.83)
Escherichia coli	such as ATCC 8739 (NCIMB 8545, CIP 53.126)
Bacillus subtilis	such as ATCC 6633 (NCIMB 8054, CIP 52.62)
Candida albicans	such as ATCC 10231 (NCPF 3179, IP 48.72)
Aspergillus niger	such as ATCC 16404 (IMI 149007, IP 1431.83)

Use buffered sodium chloride-peptone solution pH 7.0 to make reference suspensions containing about 100 colony-forming units per millilitre. Use the suspension of each of the micro-organisms separately as a control of the counting methods, in the presence and absence of the product to be examined. When testing the membrane filtration method or the plate-count method, a count of any of the test organisms differing by not more than a factor of five from the calculated value from the inoculum is to be obtained. When testing the most-probable-number method the calculated value from the inoculum is to be within the 95 per cent confidence limits of the results obtained. To test the sterility of the medium and of the diluent and the aseptic performance of the test, carry out the method using sterile sodium chloride-peptone solution pH 7.0 as the test preparation. There must be no growth of micro-organisms.

INTERPRETATION OF THE RESULTS

The bacterial count will be considered to be equal to the average number of colony-forming units found on agar medium B. The fungal count will be considered to be equal to the average number of colony-forming units on agar medium C. The total viable aerobic count is the sum of the bacterial count and the fungal count as described above. If there is evidence that the same types of micro-organisms grow on both media this may be corrected. If the count is carried out by the most-probable-number method the calculated value is the bacterial count.

When a limit is prescribed in a monograph it is interpreted as follows:

10^2 micro-organisms: maximum acceptable limit: 5×10^2,

10^3 micro-organisms: maximum acceptable limit: 5×10^3, and so forth.

If a sampling plan such as the three-class sampling plan for example, is used, proceed as follows:

Calculate the total viable aerobic count separately for each of the five samples. The substance or preparation passes the test if the following conditions are fulfilled:

(i) none of the individual total viable aerobic counts exceeds the prescribed limit by a factor of ten or more (i.e. no "unacceptable samples"),

(ii) and not more than two of the individual total viable aerobic counts are between the prescribed limit and ten times this limit (i.e. no more than two "marginal samples").

The solutions and culture mediums recommended are described in the general chapter *2.6.13*.

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2.6.13. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS (TEST FOR SPECIFIED MICRO-ORGANISMS)

In this general method the use of certain selective media is proposed. A feature common to all selective media is that sub-lethally injured organisms are not detected. As sub-lethally injured organisms are relevant for the quality of the product a resuscitation must be included in examination procedures that rely on selective media.

If the product to be examined has antimicrobial activity this must be adequately neutralised.

Enterobacteria and certain other gram-negative bacteria

Although the test has been designed to detect bacteria belonging to the family of Enterobacteriaceae, it is recognised that other types of organisms (e.g. *Aeromonas, Pseudomonas*) may be recovered.

Detection of bacteria. Prepare the product to be examined as described in the general method *2.6.12*, but using broth medium D in place of buffered sodium chloride-peptone solution pH 7.0, homogenise and incubate at 35-37 °C for a time sufficient to revive the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 h but not more than 5 h). Shake the container, transfer the