Research Article

Evaluation of the Virucidal Effect by Contact with Water-insoluble Substances: The Case of Xibornol

Marco Verani[1*], Ileana Federigi[1], Cristina Bigini[2], Fabrizio Nannipieri[2], Stefano Ceccanti[2], Sandra Vecchiani[2], Annalaura Carducci[1]
[1]Hygiene and Environmental Virology Laboratory, Department of Biology, University of Pisa, Pisa, Italy.
[2]Abiogen Pharma S.p.A., Pisa, Italy.
***Corresponding author's E-mail:** marco.verani@unipi.it

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ABSTRACT

The virucidal activity of xibornol has been known since the 70s, but its determination is primarily based on few clinical data. Experimental quantitative estimation *in vitro* is limited by the absence of appropriate test neither the European nor the American Pharmacopoeia; however some recent standards, concerning antiseptics, could be utilized for this purpose. The tests were carried out by following the European Standard EN 14476, which foresees contact test between viruses and 3 different concentrations of xibornol, in two experimental conditions and viral titer estimation by cell culture method. The tested viruses were chosen among those ones most closely responsible of upper respiratory tract infections: Human Adenovirus, Human Rhinovirus and Human Corona virus. For the commercial concentration (3%), the results show a common trend for all the viruses with an estimated titer that reached values below 1 log DCP_{50}/ml after each condition contact test. The reduction was greater in clean conditions with average value of 3 logs, compared to the test in dirty conditions (2 log). The data confirm the virucidal action of xibornol and show, for the first time, a quantitative variation in disinfection efficiency depending on contamination scenario.

Keywords: Xibornol, virucidal activity, virus, cell culture tests.

INTRODUCTION

The virucidal activity of 6-isobornyl-3-4-xylenol (i.e. xibornol) has been known since the 1970s, and has been repeatedly documented for about a decade, however its determination is primarily based on clinical data and partly on data obtained from qualitative studies *in vitro* according to the state-of-the-art of the era (assays on embryonated eggs).For this reason, it was considered appropriate to reassess a suspension formulated for oral hygiene by means of *in vitro* tests that investigate more viruses, which are representative of the main pathogens responsible for infections of the mouth and upper airways, and to quantify the virucidal activity of xibornol. Currently, unfortunately, neither the European nor the American Pharmacopeia provides appropriate tests for determining the virucidal activity of drugs, so we considered international standards issued for determining the virucidal activity of antiseptics[1].Although in the past the virucidal activity for these devices was determined according to national standards or alternative methods to these, such as "Viraden", the activity has long been evaluated by reference to the standards published by the European Committee for Standardization[2-3].We have decided to adopt these standards as a reference and in particular, the case that specifies test methods and minimum requirements for the evaluation by means of tests in suspension: EN 14476-2007[4]. In fact, this regulation is relatively recent; it has already been implemented by various national standard bodies, including the Italian one, and is applicable to non-filterable samples, such as xibornol. The *in vitro* method indicated consists of a challenge with the biocidal product under controlled conditions (duration and exposure conditions) and in determining the consequent reduction of the viral titer. The method also includes tests to ensure that the results are not affected by interferences such as cytotoxicity on cell cultures used for the titration. Compared to the standard, the following changes have been made: elimination of alcohol from the formula because it has virucidal activity and the use of a neutralizer for diluting the product, to eliminate the cytotoxic effects[5-6]. The virucidal activity of xibornol was then determined by extrapolation of the results obtained at different dilutions and in the absence of synergy with alcohol.

MATERIALS AND METHODS

The *in vitro* investigations were carried out using tests that followed, as far as possible, the European standard UNI EN 14476 entitled "Virucidal activity of chemical disinfectants or antiseptic products for instruments, surfaces or hands, which form a physically stable homogeneous preparation when diluted with hard water or with water", which involves the use of *in vitro* tests based on cell cultures. The viruses on which to test the active ingredient were selected from those most closely responsible for infections of the upper respiratory tract: Human Adenovirus, Human Rhinovirus and Human Coronavirus[7]. The tests were conducted with ATCC strains for each of these viral agents (VR-5 Human Adenovirus Type 5 Strain Adenoid 75; VR-286, Human Rhinovirus 13 Strain 353; VR-740 Human Coronavirus Strain 229E), isolated on two cell lines: HeLa (ATCC CCL-2), susceptible to infection by Human Adenoviruses and Human Rhinovirus and MRC-5 (ATCC CCL-171), susceptible to infection by Human Coronavirus.

Viral titration

The viruses were titrated using the Karber method, which allows detection of the cytopathogenic dose that affects 50% of the inoculated cells (DCP_{50})[8].The procedure followed is the standard one recommended by the UNI EN 14476 and involves the seeding of viruses in microplates with cytopathic effect evaluation after 5 days.

Evaluation of the toxicity of xibornol against the cell line

The tests to check the toxicity of the disinfectant on the cell lines were carried out according to the protocol of the standard UNI EN 14476.As a premise to these, however, it should be emphasized that xibornol is soluble in alcohol (ethanol), which is already well known to have toxic effects on cell cultures. The disinfectant was tested at three different dilutions: 8%, 3% (the commercial dilution) and 0.5%. For each test, 2 ml of suitably prepared hard water was mixed with 8 ml of disinfectant for each dilution. These mixtures were serially diluted with culture medium and serum, and the dilutions (1:10; 1:100; 1:1000) were seeded in flasks with a layer of confluent cells. After 1 h of contact, the mixtures were recovered, culture medium and 2% serum were added and the flasks were incubated at 37°C. The flasks were observed under the microscope at different times (after 1 h and then daily for 5 days or until the appearance of apparent toxic effects on the cells). A maximum of 5 days of observation was envisaged, to take into account the observation times required for the viral titrations.

Detoxification tests

For the cell lines, regarding the toxicity to xibornol and to the ethanol in which it was diluted, it was decided to use disinfectant preparations where the active ingredient was diluted in solutions other than water, with the aim of being able to test the conditions involving both solutions and suspensions. Even for these new mixtures, it was necessary to test the toxicity for the cell lines following the same method of the previous tests. The disinfectant was tested in solution (xibornol + Labrasol) and in suspension (xibornol + Avicel RC 591), each at different dilutions: 8%, 3% (the commercial dilution) and 0.5%. For each test, mixtures were assayed that were composed of 2 ml of suitably prepared hard water and 8 ml of disinfectant for each dilution, both in suspension and in solution. These mixtures were serially diluted with culture medium and serum, and dilutions (1:10; 1:100; 1:1000) were seeded in flasks with a layer of confluent cells. After 1 h of contact, the mixtures were recovered, culture medium and 2% serum were added and the flasks were incubated at 37°C. The flasks were observed under the microscope at different times (after 1 h and then daily for 5 days or until the appearance of apparent toxic effects on the cells). A maximum of 5-7 days of observation was envisaged, to take into account the observation times required for the viral titrations. Both Labrasol and Avicel RC 591 were inoculated in two separate flasks to indicate any effects on the cells independently from xibornol.

Laboratory tests of virucidal activity

The tests were performed according to the European standard UNI EN 14476, considering two conditions: “clean” and “dirty”. For each of the three viruses, three replicates were performed, with mixtures of 10 ml consisting of 1 ml of Labrasol (“clean” tests) or 1 ml of fetal bovine serum (using for “dirty” tests); 8 ml of disinfectant for each dilution (8%, 3%, 0.5%) at non-toxic concentrations for the cell cultures, corresponding to 0.008 g/100 ml; 0.03 g/100 ml and 0.05 g/100ml, respectively, diluted in Labrasol, and 1 ml of virus of known titer. For the tests performed in “dirty” conditions, the erythrocytes were not mixed together with the fetal bovine serum, differently to that indicated by the European standard, because of the interference effect that they have on the cell lines used in the tests. Overall, for each agent, six disinfectant-virus combinations were tested (three for the clean tests and three for the dirty tests). Furthermore, for each replicate, a negative control was added, consisting of 1 ml of virus and 9 ml of Labrasol (“clean” tests) or 8ml Labrasol + 1 ml fetal bovine serum (“dirty” tests). After a contact time of 15 minutes, the mixtures were analyzed to evaluate the residual viral titer by titration method.

Statistical Analysis

The data obtained were analyzed with the program Microsoft Excel for evaluating the means and total logarithmic standard deviations, and for the graphical representation of the trend and of the viral abatement to xibornol concentrations.

RESULTS AND DISCUSSION

Viral titration

The titers obtained were: 1.2×10^5 DCP_{50}/ml for Human Adenovirus 5, 7.5×10^5 DCP_{50}/ml per Human Coronavirus and 1.5×10^4 DCP_{50}/ml for Human Rhinovirus.

Evaluation of the toxicity of xibornol against the cell line HeLa

The toxicity tests revealed a widespread xibornol toxic effect for cells lines already after 1 contact day for all the dilutions. From these results, it was necessary to include a detoxification step of the virus-disinfectant mixture before seeding on cells. Furthermore, the insolubility in water of xibornol prevented, even at higher dilutions, the exact use of the procedure relative to the European standard UNI EN 14476.In fact, dilution with hard water as suggested by this standard, produced a large amount of precipitate. The toxicity of the ethanol (well known) used for the dilution of the disinfectant was added to the toxicity of the xibornol itself.

Detoxification tests

The results reported in Table 1 take into account the toxic effects that are seen before 5 days.

Table 1: Detoxification trials results

	Dilutions of the mixture	Toxic Effect within 5 days	Start of the Toxic Effect
Solution (xibornol + Labrasol) 8%	No dilute	YES	1 h
	1:10	YES	1 h
	1:100	YES	1 h
	1:1000	YES	1 h
Solution (xibornol + Labrasol) 3%	No dilute	YES	1 day
	1:10	YES	1 h
	1:100	YES	1 h
	1:1000	NO	7 days
Solution (xibornol + Labrasol) 0.5%	No dilute	YES	1 day
	1:10	YES	2 days
	1:100	NO	7 days
	1:1000	NO	7 days
Labrasol	No dilution	NO	7 days
Sospension (xibornol + Avicel RC 591) 8%	No dilute	YES	1 h
	1:10	YES	1 h
	1:100	YES	1 h
	1:1000	YES	1 h
Sospension (xibornol + Avicel RC 591) 3%	No dilute	YES	1 h
	1:10	YES	1 h
	1:100	YES	1 h
	1:1000	YES	1 day
Sospension (xibornol + Avicel RC 591) 0.5%	No dilute	YES	1 h
	1:10	YES	1 h
	1:100	YES	2 days
	1:1000	NO	6 days
Avicel RC 591	No dilution	YES	1 h

From these results it was found that both the suspension and the solution are more soluble comparing to the mixtures of xibornol previously provided. However, different toxic effects can still be noted:

Solution: The toxic effect for each dilution with culture medium, which is predominantly an aqueous solution, seems to decrease in relation to the concentration of the disinfectant. It should be noted that Labrasol in itself is not toxic.

Suspension also in this case, the toxic effect for each dilution is reduced in proportion to the concentration of the disinfectant. However, it is noted that in this case Avicel RC 591 in itself is toxic.

From the obtained data, it was suggested to carry out the tests on the virucidal activity exclusively considering the solutions through variation of the protocol UNI EN. In particular, replacing the hard water and the culture medium, it was decided to directly use Labrasol as the diluent of the disinfectant, thus avoiding the toxic effect that defines the mixing with these aqueous solutions and, therefore, being able to test less diluted preparations (Table 2).

Laboratory tests of virucidal activity

The mean results are reported in the following tables and are sorted by virus and type of test (Tables 3-4-5). The logarithmic viral abatement, relating to the different working concentrations and conditions, for Human Adenovirus 5, resulted in a range from 2.4 to 3.3 in clean conditions and constant for dirty ones with a mean of 2 log reduction. Human Coronavirus abatement resulted higher in clean conditions (3.4-3.6) than in dirty ones (2.3-2.5); instead Human Rhinovirus reduction seemed to not be influenced by conditions. These data confirmed the protection role against disinfection for viruses of particles presented in solution.

Table 2: Data on the cell cultures obtained with Labrasol.

	Dilutions of the mixture with Labrasol	Toxic Effect within 5 days
Solution (xibornol + Labrasol) 8%	No dilute	YES
	1:10	YES
	1:100	YES
	1:1000	NO
Solution (xibornol + Labrasol) 3%	No dilute	YES
	1:10	YES
	1:100	NO
	1:1000	NO
Solution (xibornol + Labrasol) 0.5%	No dilute	YES
	1:10	NO
	1:100	NO
	1:1000	NO

Aggregation in particle is one of the most important factors that enhance virus survival in different environmental matrices, such as water system. In water treatment plants, many authors reported that the presence of organic matter reduces enteric virus removal, because the absorption to the particles that were not eliminated during clarification and filtration protects viruses from chemical disinfection[9-10].

Graphical analysis and graphical trends

The obtained data with the "working" concentrations in logarithmic values for each replicate test, are reported on scatter plots using Microsoft Excel program (Figures 1-2). In these graphs, the trends of the viral titers and of the viral abatement are visualized and, using the equations of the curves obtained, these values were extrapolated at the commercial concentration (3%).

Graphical trends

The obtained data show a trend of the viral titer that reduces with logarithmic values less than 1, in every condition, for the commercial concentration (Figure 1). For Human Adenovirus 5, the "clean" tests data range to an extrapolated value of 0.08 log DCP_{50}/ml, while for the "dirty" tests, to of 0.17 log DCP_{50}/ml. Human Coronavirus "clean" tests revealed at the commercial concentration a value of 0.33 log DCP_{50}/ml and for the "dirty" ones, of 0.55 log DCP_{50}/ml. At the commercial concentration, for Human Rhinovirus, the "clean" tests data permitted to extrapolate a titer of 0.27 log DCP_{50}/ml, while for the "dirty" tests, the valued obtained was 0.11 log DCP_{50}/ml.

Table 3: Human Adenovirus 5. "Clean" data obtained - Mean viral titer after 15 min of contact (Log DCP_{50}/ml ± Standard Deviation) and logarithmic abatement.

	Dilution used	"Working" concentrations (g/100 ml)	Clean		Dirty	
			Viral titer	Logarithmic abatement	Viral titer	Logarithmic abatement
Positive control			4.4± 0.1		3.1 ± 0.2	
8% Xibornol Solution	1:1000	0.008	2± 0.8	2.4	1.1 ± 0.6	2
3% Xibornol S olution	1:100	0.03	1.3± 1	3.1	1.2± 0.6	1.9
0.5% Xibornol Solution	1:10	0.05	1.1± 0.1	3.3	0.8± 0.5	2.3

Table 4: Human Corona virus. "Clean" data obtained - Mean viral titer after 15 min of contact (Log DCP_{50}/ml ± Standard Deviation) and logarithmic abatement.

	Dilution used	"Working" concentrations (g/100 ml)	Clean		Dirty	
			Viral titer	Logarithmic abatement	Viral titer	Logarithmic abatement
Positive control			5.2± 0.2		4.1 ± 0.2	
8% Xibornol Solution	1:1000	0.008	1.8± 0.7	3.4	1.7 ± 0.7	2.3
3% Xibornol Solution	1:100	0.03	1.4± 0	3.8	1.5± 0.6	2.6
0.5% Xibornol Solution	1:10	0.05	1.6± 0.3	3.6	1.6± 0.4	2.5

Table 5: Human Rhinovirus. "Clean" data obtained - Mean viral titer after 15 min of contact (Log DCP_{50}/ml ± Standard Deviation) and logarithmic abatement.

	Dilution used	"Working" concentrations (g/100 ml)	Clean		Dirty	
			Viral titer	Logarithmic abatement	Viral titer	Logarithmic abatement
Positive control			4.2± 0.3		3.8± 0.1	
8% Xibornol Solution	1:1000	0.008	2± 0.1	2.2	1± 0.1	2.8
3% Xibornol Solution	1:100	0.03	1.3± 0.5	2.9	0.7± 0.1	3.1
0.5% Xibornol Solution	1:10	0.05	1.2± 0.4	3	0.8± 0.1	3

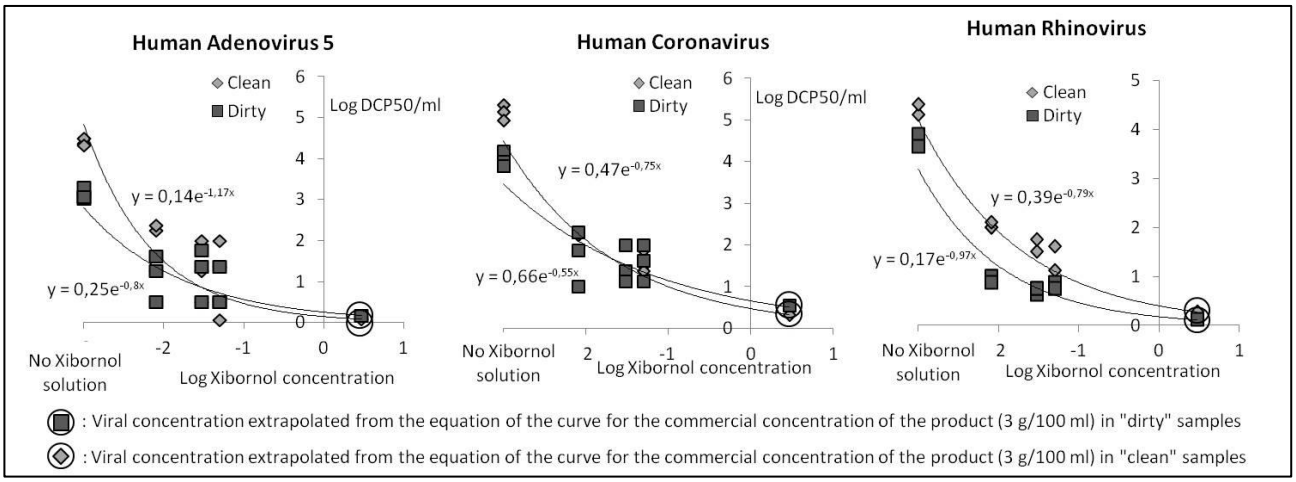

Figure 1: Graphical trend

Graphical abatement

Human Adenovirus 5 abatement at commercial concentration was graphical estimated of 6.21 log for "clean" tests and of 2.7 Log for "dirty" ones. Human Coronavirus abatement was 4.8 at 3% concentration for "clean" tests, and, for the "dirty" tests, the value resulted 3 Log. Human Rhinovirus for the "clean" tests, showed an abatement of 6.51 while the extrapolation for "dirty" tests was 3.5 Log (Figure 2).

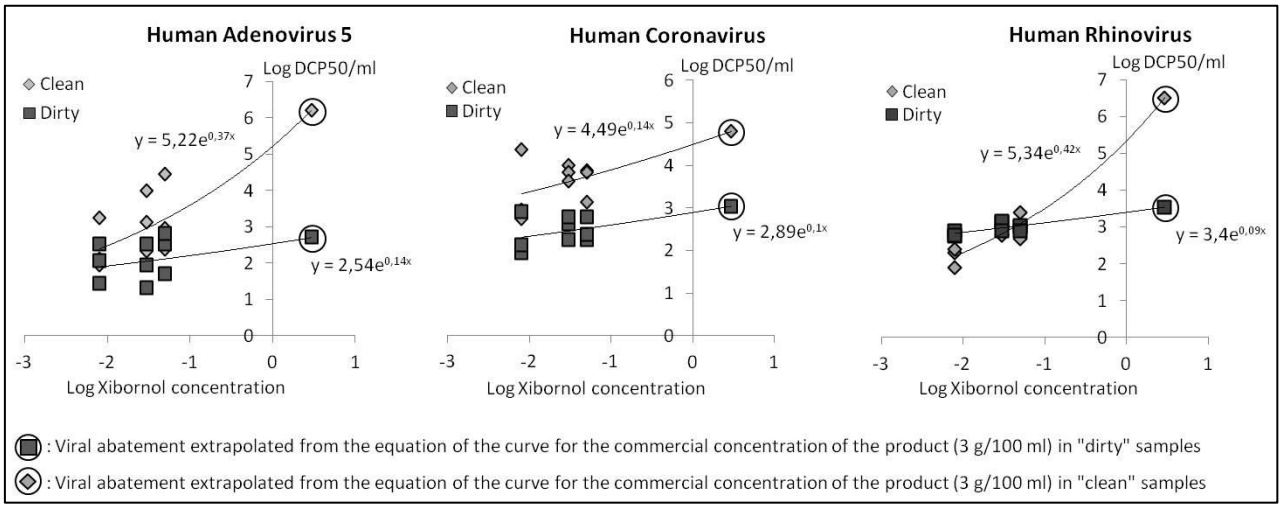

Figure 2: Graphical abatement

CONCLUSION

The data, despite being obtained with dilutions of the disinfectant concentrations required to prevent the toxic effect on cell lines, confirm the virucidal action. Starting from this premise, although the tests carried out made use of validation protocols of disinfectants on the environment, and in relation to the data related to the "dirty" conditions, it is conceivable to extend the study with a view to creating a possible clinical trial of the product to also verify its effectiveness on the oral mucous membranes.

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