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Antimicrobial activity of a formulation for the low temperature disinfection of critical and semi-critical medical equipment and surfaces

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SUMMARY

The antimicrobial activity of the disinfectant formulation UMONIUM[38] (Isopropyl-tridecyl-dimethyl-ammonium; Huckert's International, Nivelles, Belgium) planned for the low temperature disinfection of critical and semi-critical medical equipment and surfaces was evaluated under clean and dirty experimental conditions (high and low concentrations of organic material). The formulation was obtained by a synergic combination of three different active compounds, two alcohols and a quaternary ammonium. The anti-mycobacterial (*Mycobacterium avium* and *Mycobacterium terrae*), and antiviral (Poliovirus type 1, Adenovirus type 5, hepatitis B virus, and human immunodeficiency virus type 1) activities of this formulation were addressed using suspension assays. In addition, surface assays were also used to test the antibacterial (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus hirae*) and antimycotic (*Candida albicans* and *Aspergillus niger*) activities. The data document the dynamics of the antimicrobial activity under in vitro controlled conditions and highlight the relatively low influence of organic material on its activity.

KEY WORDS: Low-temperature disinfection, Synergic compounds, Antimicrobial activity

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INTRODUCTION

The theoretical concepts and practices of disinfection vary considerably according to the different applications. As a direct consequence, the methods used for testing the activity of disinfectant formulations should take this diversity into account and enable the characteristics and the quality of each product to be verified under different experimental and application conditions. Indeed, a series of factors are involved in the activity of a disinfectant formulation, including:

1) the intrinsic activity of the individual compounds;
2) the nature and the physical state of the microrganisms;
3) the physical and chemical environment of disinfection (e.g. the concentration of the microbial agent, the temperature, the pH, and the presence of organic material).

High efficacy against a wide range of bacteria, yeast, molds, and viruses is currently required for modern disinfectant formulations, in parallel with low toxicity, low ecologic risk, and no sign of resistance during long-term use. Currently, a possible strategy to satisfy these requirements is to include synergic combinations of antimicrobial agents in disinfectant formulations. In fact, when the disinfectant agents show synergic activity, the concentrations of the individual compounds may be reduced (with lower toxic and ecologic risk),

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the risk of resistance during long-term use is minimized, and the range of antimicrobial activity is usually increased (Lehmann *et al.,* 2001). A large number of combinations of active compounds have been tested and used for different applications; in some cases, synergism is obtained with substances that have little or absent antimicrobial activity *per se* at the used concentration, but is greatly enhanced when included in a synergic combination. Notably, the demonstration of a synergic improvement in the activity of two or more known compounds is patentable, if not already described (Grebe *et al.,* 1991).

A crucial aspect of a correct disinfection is the interfering activity of organic material.

Depending on the chemical nature of the disinfectant formulation, the interfering properties of organic material are normally due:

1) to chemical reactions with loss of active product;
2) to surface absorption of the compound, with consequent drop of available disinfectant agent (Best *et al.,* 1990).

To evaluate the role of this interference under controlled experimental conditions, blood, serum, bovine albumin and yeast extract have been used in most assays. It was observed that the chemically reactive (e.g. acids and phenols) and the oxidizing compounds (e.g. iodine and hypoclorides) are strongly inhibited in the presence of organic material (Cremieux *et al.,* 2001), thus emphasizing the need for disinfectants that are minimally influenced under these conditions.

In the present study, we evaluated the antimicrobial activity of a disinfectant formulation (UMONIUM^{38}; Isopropyl-tridecyl-dimethyl-ammonium; Huckert's International, Nivelles, Belgium) obtained by a synergic combination of three different active compounds. The antibacterial (including anti-mycobacterial), antiviral and anti-mycotic activity of the formulation under study was evaluated under clean and dirty experimental conditions.

MATERIALS AND METHODS

Disinfectant formulation

The disinfectant formulation under study, UMONIUM^{38} (Isopropyl-tridecyl-dimethyl-ammonium; Huckert's International, Nivelles, Belgium), is a synergic and balanced combination of two alcohols and a quaternary ammonium (Isopropyl alcohol, benzalkonium chloride, and tridecyl ceteth alcohol, pH 7.0) at a concentration of 32g/100 ml. The formulation was obtained directly from the manufacturer (Lot 04H03I13) and tested after dilution using distilled water (concentrations tested: 2.5% and 0.5%). These dilutions are normally used in the low temperature disinfection of critical and semi-critical medical equipment and surfaces.

Evaluation of the antimicrobial activity under clean and dirty experimental conditions

In the present study, the antiviral effect of UMONIUM^{38} was assayed at 20°C under dirty (final concentration in the assay: 3.0 g of bovine serum albumin plus 3.0 ml erythrocytes per litre of water) and clean (final concentration in the assay: 0.3 g of bovine serum albumin per litre of water) conditions. According to the European Standards followed in the present analysis, clean conditions are conditions representative of environments or surfaces that have received a cleaning programme and/or are known to contain minimal levels of organic materials; dirty conditions are conditions representative of surfaces which are known to or may contain organic materials.

Assays to test the antimicrobial activity of UMONIUM38

The antiviral, antibacterial and antimycotic activities of the disinfectant formulation under study were evaluated in the present study. Briefly, the activity against two non-enveloped virus (Poliovirus type 1 and Adenovirus type 5) was tested using a suspension assay according to the European Standard EN14476:2002 (phase 2; step 1). Following the methodology reported in the same standard, we also tested the activity against the human immunodeficiency virus type 1 (HIV-1). The activity against hepatitis B virus (HBV) was tested using a recently developed cell culture model system (Payan *et al.,* 2001; Payan *et al.,* 2004), with minimal modification. The mycobactericidal activity was evaluated using *Mycobacterium avium* and *Mycobacterium terrae,* following the procedure described in European Standard EN14348:2005. Finally, quantitative non-porous surface tests were used to evaluate the antibacterial (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus au-*

reus, and *Enterococcus hirae*) and antimycotic (*Candida albicans* and *Aspergillus niger*) activity of UMONIUM[38], following European Standard EN13697:2001.

RESULTS

Antiviral activity: poliovirus and adenovirus

The stock virus suspensions were prepared using both Adenovirus type 5 (ATCC VR5) and Polivirus 1 Sabin strain. Virus titers of the virus suspensions (expressed as negative Log_{10} of the IC_{50} end point per ml) were as follows: Adenovirus 5 -8.03; Poliovirus 1 -8.20. To evaluate the lowest UMONIUM[38] concentration unable to generate morphological alterations of the cells, 90% confluent cell monolayers were treated using the disinfectant formulation (2.5% and 0.5% in water, respectively) from undiluted to 10^{-4}. No cytotoxic effect could be observed in the assay of the 0.5% concentration (from undiluted to 10^{-2}); the 2.5% concentration tested non cytotoxic at a concentration of 10^{-1}. Under these conditions both disinfectant preparations (2.5% and 0.5%) were used undiluted, since the sample preparation after incubation is subjected to 10-fold dilution before infection of cell monolayers. To test the virucidal activity of UMONIUM[38], clean and dirty experimental conditions were used. The other parameters were as follows: dilutions in water of the disinfectant formulation under study, 2.5% and 0.5%; contact times 1, 10, 20 and 60 minutes; temperature 20°C. A reduction of the virus titers higher than 4 Log_{10} required to document the virucidal activity of a disinfectant formulation was obtained using 2.5% UMONIUM[38] for 10 minutes or more under dirty conditions, and for 1 minute or more under clean

TABLE 1 - *Antiviral activity of UMONIUM38 evaluated under dirty and clean experimental conditions using standard procedures.*

Agent	*UMONIUM[38] [1]*	*Incubations conditions[2]*	*Standard[3]*	*Time[4]*	*Drop of infectivity[5]*
Poliovirus 1	0.50%	C	EN14476:2005	20	4
Poliovirus 1	0.50%	D	EN14476:2005	20	4
Poliovirus 1	2.50%	C	EN14476:2005	1	4
Poliovirus 1	2.50%	D	EN14476:2005	10	4
Adenovirus 5	0.50%	C	EN14476:2005	20	4
Adenovirus 5	0.50%	D	EN14476:2005	20	4
Adenovirus 5	2.50%	C	EN14476:2005	1	4
Adenovirus 5	2.50%	D	EN14476:2005	10	4
HIV-1	0.50%	C	EN14476:2005	10	4
HIV-1	0.50%	D	EN14476:2005	10	4
HIV-1	2.50%	C	EN14476:2005	1	4
HIV-1	2.50%	D	EN14476:2005	10	4
HBV	0.50%	D	See Ref. 6	10	4
HBV	2.50%	D	See Ref. 6	5	4

[1]Concentration in water; [2]D: dirty, C: clean (see text: "Material and methods"); [3]European Standard or reference method followed; [4]Time (minutes) required to meet the requirements of the appropriate standard followed in the analysis; [5]Loss of virus infectivity (Log_{10}).

conditions for both viruses (Poliovirus 1 and Adenovirus 5); using the 0.5% dilution a reduction of the virus titers higher than 4 Log_{10} was observed after incubation for 20 minutes or more under clean and dirty conditions (both viruses) (Table 1).

Antiviral activity (HIV-1 and HBV)

The anti-HIV-1 activity was evaluated following the same conditions used to test the virucidal potential for unenveloped viruses. A reduction of the HIV-1 titers higher that 4 Log_{10} was obtained

TABLE 2 - *Surface assays: bactericidal and fungicidal activity of Umonium38 evaluated using a quantitative non-porous surface test (EN 13697:2001).*

Agent	*UMONIUM38* [1]	*Incubations conditions*[2]	*Time*[3]	*Drop of infectivity*[4]
P. aeruginosa	0.50%	C	15	4
P. aeruginosa	0.50%	D	60	4
P. aeruginosa	2.50%	C	15	4
P. aeruginosa	2.50%	D	15	4
E. coli	0.50%	C	15	4
E. coli	0.50%	D	60	4
E. coli	2.50%	C	15	4
E. coli	2.50%	D	15	4
S. aureus	0.50%	C	15	4
S. aureus	0.50%	D	60	4
S. aureus	2.50%	C	15	4
S. aureus	2.50%	D	15	4
E. hirae	0.50%	C	15	4
E. hirae	0.50%	D	60	4
E. hirae	2.50%	C	15	4
E. hirae	2.50%	D	15	4
C. albicans	0.50%	C	15	3
C. albicans	0.50%	D	60	3
C. albicans	2.50%	C	15	3
C. albicans	2.50%	D	15	3
A. niger	0.50%	C	15	3
A. niger	0.50%	D	60	3
A. niger	2.50%	C	15	3
A. niger	2.50%	D	15	3

[1]Concentration in water; [2]D: dirty, C: clean (see text: "Material and methods"); [3]Time (minutes) required to meet the requirements of the appropriate standard followed in the analysis; [4]Loss of infectivity (Log_{10}).

using 2.5% UMONIUM[38] for 10 minutes or more under dirty conditions, and 1 minute or more under clean conditions. Using the 0.5% dilution of the disinfectant formulation a reduction of the virus titers higher than 4 Log_{10} was observed after incubation for 10 minutes or more under clean and dirty conditions. The anti-HBV activity was evaluated using a recently optimized cell culture assay[6]; under these conditions, a 4-log reduction of infectious titers of HBV suspensions was documented after 10 minutes of incubation using a 0.5% dilution of UMONIUM[38] and after 5 minutes of incubation using a 2.5% dilution of UMONIUM[38]. The experimental conditions of the assays can be considered as "dirty" due to the presence of high concentrations of human plasma (Table 1).

Mycobactericidal activity

The mycobactericidial activity of the disinfectant formulation UMONIUM[38] was assayed using *Mycobacterium terrae* and *Mycobacterium avium* following the methodology described in European Standard EN14348:2005. In this standard, the test is performed in suspension. The inactivation factor is calculated from the ratio of the bacterial counts before and after disinfection. In our assay, solutions of mycobacteria and interfering substances were added to a sample of the product.

The mixture was maintained at 20 °C for 60 minutes, 30 minutes, 10 minutes and 5 minutes. At these contact times, an aliquot was immediately neutralized.

Finally, the number of surviving mycobacteria and the number of mycobacteria in the test suspension were determined, and the reduction of viable counts calculated. Under these conditions, the product under study is considered mycobactericidal when a log reduction of 5 or greater with both test organisms is observed, while if such results are seen for *M. terrae* only, the product is considered tuberculocidal. Following the criteria of the standard, the disinfectant formulation UMONIUM[38] at a concentration of 0.5% documented mycobactericidal activity after 60 minutes under dirty and clean experimental conditions. At a concentration of 2.5%, UMONIUM[38] showed mycobactericidal activity after 30 minutes of incubation under dirty and clean experimental conditions.

Surface assays (bactericidal and fungicidal activity)

The bactericidal and fungicidal activities of UMONIUM[38] were evaluated using a quantitative non-porous surface test (EN 13697:2001). The assay was specifically designed to test disinfectant formulations, simulating practical application conditions. Briefly, for the bactericidal activity, the assay evaluates the capability of a product to produce at least a 10^4 reduction in the number of viable cells belonging to reference strains of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Enterococcus hirae*, while for the fungicidal activity, it evaluates the capability of a product to produce at least a 10^3 reduction in the number of viable fungi belonging to reference strains of *Candida albicans* and *Aspergillus niger*. The product under evaluation was tested at 20°C for 5 minutes, 10 minutes, 15 minutes and 60 minutes. At a concentration of 2.5% UMONIUM[38] determined a reduction of 4 log_{10} or more for all the bacterial strains tested after 15 minutes or more of incubation, under dirty and clean conditions. At a concentration of 0.5% UMONIUM[38] determined a reduction of 4 log_{10} or more for all the bacterial strains tested after 15 minutes or more of incubation under clean conditions and after 60 minutes of incubation under dirty conditions. In the assay of the fungicidal activity, at a concentration of 2.5% UMONIUM[38] determined a reduction of 3 log_{10} or more for the fungi tested after 15 minutes of incubation, under dirty and clean conditions. When diluted at 0.5% UMONIUM[38] determined a reduction of 3 log_{10} or more after 15 minutes or more of incubation under clean conditions and after 60 minutes of incubation under dirty conditions.

DISCUSSION

Design and optimization of disinfectant formulations that meet the growing requirements for ever-higher standards of infection control is currently a crucial challenge (De Lorenzi *et al.*, 2006; Murdoch *et al.*, 2006; Kramer *et al.*, 2006; Sacchetti *et al.*, 2007). Use of synergic combinations of antimicrobial agents is a possible strategy to satisfy some requirements, since these conditions allow the maintenance of some advantages

(multiple mechanisms of activity, different targets, lower risk of resistance in prolonged treatments), while the disadvantages are minimized by the low concentrations of individual compounds (lower toxicity).

In the present study, we analyzed the antimicrobial activity of a synergic and balanced combination of two alcohols and a quaternary ammonium (isopropyl alcohol, benzalkonium chloride, and tridecyl ceteth alcohol) at a concentration of 32 g/100 ml (UMONIUM^{38}; Huckert's International, Nivelles, Belgium). The formulation was tested after dilution using distilled water, at concentrations of 2.5% and 0.5%), under clean and dirty experimental conditions. These dilutions are normally proposed for the low-temperature disinfection of critical and semi-critical medical equipment and surfaces.

The antiviral activity of UMONIUM^{38} was assayed using the procedure described in European Standard EN14476:2005. An antiviral activity reaching a drop of 4 log in infectivity of virus suspensions (Adenovirus type 5 and Poliovirus type 1) was observed for both viruses after 10 minutes and 1 minute using the product at a concentration of 2.5%, under dirty and clean incubation conditions, respectively.

Similarly, the anti-HIV-1 activity, assayed and evaluated following the same procedure and requirements, was documented to occur within 10 minutes and 1 minute using the product at a concentration of 2.5%, in the presence or absence of high concentrations of interfering substances, respectively.

The anti-HBV activity was tested using a cell culture method in the present study. Indeed, the evaluation of the activity of disinfectant formulations has been strongly hampered in the last few decades by lack of reliable cell culture model systems. HBV infects efficiently the chimpanzee, but the chimpanzee is a protected species and assays using this model are highly expensive. A closely related virus, the duck hepatitis B virus (DHBV) can be used in an infectivity assay to test disinfectants (Deva *et al.,* 1996). Unfortunately, significant differences exist in the chemical composition of the surface of HBV and DHBV (Thraenhart and Jursch, 2001), thus suggesting that a direct assumption of the DHBV-based data for HBV is not possible.

A morphological assay (designated morphological alteration and disintegration test; MADT) has been proposed to evaluate the activity of disinfectant formulation on HBV infectivity. This method evaluates the physical integrity of infectious HBV particles using electron microscopy and assumes that the physical destruction of the virus particle is correlated with the inactivation of the infectivity (Thraenhart and Jursch, 2001). This method is time-consuming, requires electron microscopy and the results are strongly dependent on user experience. More recently, a novel cell culture-based method has been proposed to test the activity of disinfectants against HBV (Payan *et al.,* 2004). In the present study, we used a modification of this culture method that parallels perfectly other assays of the virucidal activity for different pathogens, allowing the evaluation of infectivity of treated and untreated virus suspensions. The data show that an infectivity reduction of 4 log is obtained after 10 minutes of incubation using the product at a concentration of 0.5 %, under dirty conditions. Overall, the virucidal activity of the formulation under study at the concentration of 2.5% in water was efficient and minimally influenced by the presence of high concentrations of organic material.

The mycobactericidal activity of UMONIUM^{38} has been assayed using a suspension test. Infection control measures for mycobacterial infections are currently crucial in industrialized countries and in the Third World. Similarly, the evaluation of the activity of a disinfectant formulation specifically designed for environmental disinfection under conditions that mimic its use (surface assays) is central in the understanding of its characteristics and mode of action. Of note, UMONIUM^{38} at the concentration of 2.5% was not influenced by the incubation conditions (clean and dirty) in its bactericidal and fungicidal activity, thus further confirming the relatively low influence on this formulation of organic material.

The data from surface assays substantially confirm the conclusions of suspensions tests, documenting that at the concentration of 2.5%, the formulation under study shows a bactericidal and fungicidal activity after a contact time of 15 minutes irrespective of the concentration of organic material.

Overall, the present study extends our understanding of the main features of UMONIUM^{38} an-

timicrobial activity, and strongly suggests that the strategy adopted to plan this disinfectant formulation may be usefully employed for generating modern disinfectants for specific applications.

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