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Correlation between *in vitro* release from topical delivery vehicles and microbicidal activity of triclosan

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Received February 11, 2005, accepted March 8, 2005

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Pharmazie 61: 35–40 (2006)

This study reports the formulation, stability, in vitro release and microbicidal activity of a cream, emulsion, foot gel, cover stick and after sun spray containing triclosan. Triclosan is a broad-spectrum antimicrobial agent with activity against a wide range of both gram-negative and gram-positive bacteria that has found increasing popular use in personal care products. These products were stable for up to 3 months when stored at 5, 25, and 40 °C. Antimicrobial zone inhibition tests showed that that was a liner relationship, $R^2 > 0.92$, between the release of triclosan from these products and the size of the inhibition zones. This means the *in vitro/in vivo* correlation for these products was good and that release studies can be used to predict the antimicrobial activity of triclosan.

1. Introduction

Triclosan is a diphenyl ether (bis-phenyl) derivative, known as either 2,4,4′-trichloro-2′-hydroxydiphenyl ether or 5-chloro-2-(2,4-dichlorophenoxy) phenol. It is a broad-spectrum antibacterial/anti-microbial agent (Savage 1971). As a result of its bacteriostatic activity against a wide range of both gram-negative and gram-positive bacteria it has found increasing and recently popular use in personal care products, i.e. toothpaste, deodorant soaps, deodorants, antiperspirants and body washes, detergents, dish washing liquids, cosmetics and anti-microbial creams, lotions and hand soaps (Waaler et al. 1993; Nissan and Ochs 1998; Loftsson et al. 1999; Jones et al. 2000). It is also used as an additive in plastics, polymers and textiles to give these materials antibacterial properties (Lu et al. 2001). Triclosan shows bacteriostatic activity at low concentrations against the majority of bacterial strains, but is almost insoluble in water, i.e. soluble to the extent of only 10 ppm, but readily soluble in alkaline solutions and in the majority of organic solvents (Savage 1971; Grove et al. 2003; Taylor et al. 2004).

In recent studies it was reported that there is a direct correlation between the antimicrobial activity of this water insoluble microbicide and the solubilizing agents used to incorporate it into aqueous delivery vehicles (Grove et al. 2003; Taylor et al. 2004). These studies report that in surfactant solutions, increasing the surfactant : triclosan ratio causes a decrease in antibacterial efficacy, consistent with a model for micellar solubilization where the micelle binding constant, $K = \frac{[X]}{[cW]}$ increases with decreasing triclosan concentration in the micelles, [X], resulting in decreased concentration of bioavailable triclosan in the water (continuous) phase [cW]. The rapid and potent reduction of bacteria reported in these studies were surprising and support the existence of a non-specific mode of action for triclosan (Taylor et al. 2004).

In addition to the effect of solubilizing agents of the microbicidal effect, the rate at which triclosan is released (available) from semi-solid and liquid delivery systems would also influence its antimicrobial activity. This is especially important since the evaluation of *in vitro* release of actives from semisolid preparations has received much attention in recent years (Borsadia et al. 1992; Rege et al. 1998). Release is a function of several characteristics within the semi-solid, so that the constancy of release from one batch to another implies that the manufacturing process is the same and if there is a correlation between release and microbicidal activity it could also provide evidence of consistent bioactivity. This is because the uptake of triclosan by both cell walls and whole cells is due to the hydrophobic and lipophilic nature of the antibacterial agent and since the solubility of triclosan in cell components is greater than in the surrounding water-rich environment, once released from the topical vehicles it would readily penetrate microbial cells (Villalain et al. 2001; Guillen et al 2004).

Therefore, the aim of this study was to determine if there is a correlation between the rate at which triclosan is released from newly formulated, stable topical delivery vehicles and its microbicidal activity as measured by microbial zone inhibition.

2. Investigations, results and discussion

2.1. Formulation and stability of topical vehicles

This study reports the stability of and release of triclosan from five topical delivery formulations. The composition and the method of preparation for these products are given in Tables 1–5.

Table 1: Composition and method of preparation of the triclosan cream

Part	Ingredient	% (w/w)	Purpose
A	Triclosan	3.0	Microbicide
	Polyoxyl castor oils	4.0	Emulsifier
	Acid stable glyceryl monostearate	2.0	Emulsifier
	Cetyl stearyl 2-ethyl hexanoate	15.0	Emollient
	Arachis oil	5.0	Oil
	Liquid paraffin	9.0	Oil
B	Methyl hydroxybenzoate	0.3	Preservative
	Propyl hydroxybenzoate	0.2	Preservative
	Propylene glycol	3.0	Solvent
	Phosphate buffer pH 5.8	58.5	Water phase

Procedure:
Mix the ingredients of Part A and melt at 80 °C. Mix the ingredients of Part B and melt at 80 °C. Mix melted Parts A and B by stirring whilst it is cooled. Perfume and color can be added to this formulation

Table 2: Composition and method of preparation of the triclosan emulsion (lotion)

Part	Ingredient	% (w/w)	Purpose
A	Triclosan	3.0	Microbicide
	Arachis oil	50.0	Solvent
	Polyoxyethylene sorbitan monooleate	5.0	Emulsifier
	Sorbitan monooleate	5.0	Emulsifier
	Butylated hydroxyanisole	0.01	Antioxidant
	Methyl hydroxybenzoate	0.1	Preservative
	Propyl hydroxybenzoate	0.1	Preservative
B	Sorbitol	5.0	Humectant
	Phosphate buffer pH 5.8	31.8	Water phase

Procedure:
Mix the ingredients of Part A with a high-speed high-shear mixer. Similarly mix the ingredients of part B. Then mix parts A and B using the same blender for 30 min. to 1 h. Perfume and color can be added to this formulation

Table 3: Composition and method of preparation of the triclosan foot gel

Part	Ingredient	% (w/w)	Purpose
A	Triclosan	3.0	Microbicide
B	Ethanol	20.0	Solvent/ Preservative
	Citric acid buffer pH 4.6	33.0	pH control
	Propylene glycol	40.0	Solvent
C	Amorphous colloidal silicon dioxide	4.0	Gelling agent

Procedure:
Mix the ingredients of part B. Add the triclosan (part A) and stir till dissolved. Add part C to the mixture of parts A and B. Stir to form a homogenous dispersion. Set aside for gel to form. Perfume and color can be added to this formulation

Chemical stability of triclosan in these products was measured by HPLC. An example of a chromatogram is shown in Fig. 1. The retention time for triclosan was approximately 5.2 minutes. The method was linear over the range of 10–250 µg/ml, with an accuracy of 99.8%, and a precision of 1.1%. Forced degradation and peak purity analysis was used to prove that the method is stability-indicating. The samples analyzed after forced degradation had extra peaks due to breakdown of the samples, but none interfered with the triclosan peak. Diode array peak purity analysis showed that the triclosan peak was still pure. No interference was from a sample prepared from a cream placebo (Fig. 1). Small changes (5%) in the flow rate, detection wavelength, mobile phase composition and injection volume did not affect the separation. A Lichrospher® RP-18 column, 125 × 4.0 mm, 5 µm was also suitable to perform the analysis, proving that the method is robust. The same method was used for assay and release testing.

Table 4: Composition and method of preparation of the triclosan cover stick formula

Part	Ingredient	% (w/w)	Purpose
A	Triclosan	3.0	Microbicide
	Liquid paraffin	3.0	Solvent
	Amorphous colloidal silicon dioxide	0.1	Thickener
	Methyl hydroxybenzoate	0.1	Preservative
B	Mixture of acid glycol esters, tribehenin and synthetic beeswaxes	3.0	Emulsifiers
	PPG-2 myristyl ether propionate	1.5	Emollient
	Stearic acid	1.5	Oil phase
C	Color blend (mixture of titanium dioxide and iron oxides, 8 : 2 w/w)	87.8	Color and bulk

Procedure: Mix the ingredients of part A and stir till triclosan and methyl hydroxybenzoate are dissolved. Melt the ingredients of part B. Mix part A and B together until homogenous. Stir in the color blend (part C). Poor into stick moulds. Set aside to congeal. Perfume can be added to this formulation

Table 5: Composition and method of preparation of the after sun spray

Part	Ingredient	% (w/w)	Purpose
A	Triclosan	3.0	Microbicide
	Ethanol	20.0	Solvent, anesthetic, preservative
B	Propylene glycol	40	Solvent
	D-panthenol	1.0	Moisturizer, vitamin
C	Buffer pH 4.6	qs	Adjust pH to 6

Procedure:
Dissolve the triclosan in the ethanol. Dissolve the D-panthenol in the propylene glycol. Mix the ethanol and propylene glycol solutions. Prepare the buffer and add enough of the buffer to adjust the pH to 6 (approximately 36 ml of a 0.2 M acetic acid: sodium acetate buffer will adjust the pH to 6)

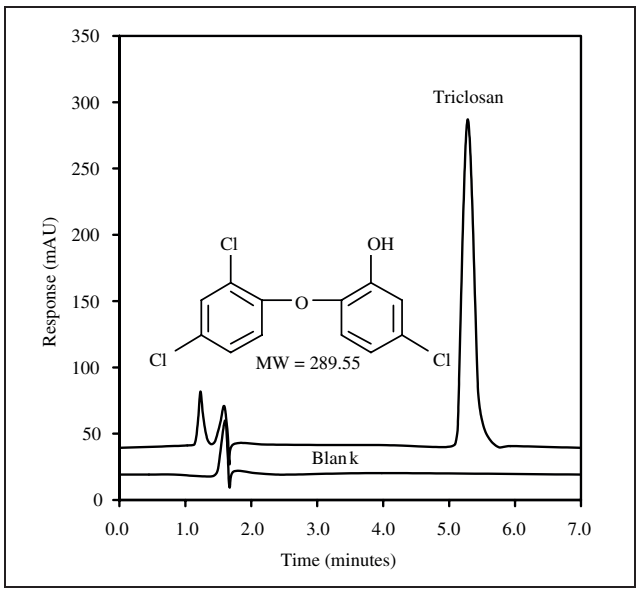

Fig. 1: HPLC chromatogram and molecular structure of triclosan

Three batches of each product containing either 1.5 or 3% triclosan was stored at 5 °C, 25 °C combined with 60% relative humidity, and 40 °C with 75% relative humidity. For the 1.5 and 3% creams, stored under these conditions for three months, there was no significant change in parti-

cle size (4–5 μm), mass loss (< 0.09% w/w), relative density ($\bar{x} = 0.96$ g/cm^3), assay (100–110%), methyl paraben (> 99.8%), propyl paraben (> 96.6%), spreadibility, preservative efficacy and visual assessment. There was a decrease in the pH of the cream from pH 6 to 5 after 3 months. For some samples the pH dropped below 5. This could be a problem because the optimum pH for triclosan antimicrobial activity is between pH 5 and 8 (Savage 1971). This change in pH was not temperature sensitive and occurred at 5, 25 and 40 °C. In addition the viscosity decreased from 1.4×10^5 cP to 9.0×10^4 cP over the three months. The decrease was more significant at 40 °C. The change in pH could explain the change in viscosity.

The emulsions (Lotion, Table 2) were also stable during the 3 months of testing because there was no significant change in pH (5.8–6.0), density ($\bar{x} = 0.97$ g/cm^3), mass loss (< 0.85% w/w), particle size (4–6 μm), assay (100–110%), preservative concentration (methyl paraben > 99.8%; propyl paraben > 98.5%), viscosity (240 ± 12 cP), visual assessment, and preservative efficacy. The lotions were also exposed to a freeze (−10 °C) thaw (40 °C) cycle of 24 h at each temperature repeated up to 10 days. No phase separation was observed indicating that the emulsion was stable to temperature fluctuations. The foot gel (Table 3) was also stable because no change in pH (5.8–6.0), density ($\bar{x} = 0.97$ g/cm^3), mass (loss < 0.4%), assay (100–101%), ethanol concentration (97–99%), preservative efficacy and visual assessment was observed during the 3 month test period at all the storage temperatures. However, a significant increase in the viscosity of the gel from 1.6×10^5 cP to 4.1×10^5 cP was observed at both low and high temperature. This increase reached a maximum after 1 month and stayed constant thereafter. The change in viscosty could be due to the stabilization of the colloidal silicon dioxide (Aerosil®) gel over time (Toricht and Eros 1977).

Recent reports on the effect of triclosan against malaria also prompted the formulation of two products, a cover stick (Table 4) and a after sun spray (Table 5), that could possibly be used as a first line of defense against the malaria parasite on the skin (Surolia and Surolia 2001; Rao et al. 2003). Both these products stayed stable at the storage temperatures during the months of testing. No viscosity, particle size, mass, visual, preservative efficacy or density changes were observed. In addition the triclosan, ethanol and preservative concentrations stayed within 95–100% of the initial test values. In addition to these tests the after sun spray, packaged in metal aerosol cans, were also tested for discharge mass ($\bar{x} = 0.0.07$ g), spray pattern and can corrosion and mass loss (0.16% w/w). These properties also did not change at the storage temperatures and during the 3 months of testing.

2.2. *In vitro release*

Release rates or flux values calculated using Higuchi's square root model are listed in Table 6. In this study ethanol : water (80% v/v) was used as the dissolution medium. This medium provided a diffusional sink for the active ingredient released from the topical formulations. The relationship of the cumulative amount release versus the square root of time was linear for all the release profiles (examples of these release profiles are shown in Fig. 2). Fitting the data this way is in accordance with Higuchi's model that assumes there is a reservoir of drug always available in the vehicle to diffuse into the receptor medium (Higuchi 1960). The values listed in Table 6 shows that the release rate from the emulsion, foot gel, cover stick, and after sun spray was not influenced by storage temperature or time of storage. When the triclosan concentration in the products was increased from 1.5 to 3% the release rate was almost doubled. This shows the concentration dependence of the release rate from these products.

However, the amount of triclosan released from the creams increased with time as shown in Fig. 2. This increase in release rate was not temperature dependent because the magnitude of the increase was not significantly different at 5, 25 or 40 °C. Most probably the increase in release rate was due to the decrease in the pH of the creams observed during stability testing. The pKa of triclosan is 7.9 at 20 °C and according to the Henderson-Hasselbalch equation an increased amount of the compound may be found in the unionized form in the water portion of the cream as the pH decrease. This increase the release rate from the creams since the unionized triclosan will diffuse rapidly into the ethanolic dissolution medium in which it is very soluble. This effect was not influenced by temperature changes. When the concentration of triclosan in the cream was doubled the increase in the release rate also doubled as shown in Fig. 2.

Table 6: Release rates, calculated using the Higuchi square root model, of triclosan from topical formulations measured with Enhancer cells using ethanol: water (80% v/v) as the dissolution medium

Formula	Conc.(%)	Release rates (μg/cm^2/min$^{0.5}$)					
		5 °C		25 °C ± 60% RH		40 °C ± 60% RH	
		Initial	3 Months	Initial	3 Months	Initial	3 Months
Cream	1.5	102.4 ± 2.5	176.6 ± 9.9	122.9 ± 6.1	190.4 ± 7.0	133.5 ± 5.1	219.9 ± 12.6
	3.0	194.1 ± 5.2	316.2 ± 10.4	215.8 ± 12.5	396.7 ± 18.5	216.1 ± 4.8	419.3 ± 15.2
Emulsion	1.5	145.3 ± 8.9	156.3 ± 11.2	148.6 ± 7.7	162.1 ± 11.2	149.3 ± 9.8	158.4 ± 13.2
	3.0	289.2 ± 7.8	312.4 ± 9.8	300.0 ± 15.8	298.4 ± 8.7	309.6 ± 12.3	312.3 ± 17.8
Foot Gel	1.5	245.3 ± 12.2	260.5 ± 9.8	255.0 ± 17.3	230.5 ± 11.3	249.8 ± 12.3	260.5 ± 10.9
	3.0	500.3 ± 18.9	490.8 ± 15.4	470.1 ± 28.9	501.3 ± 12.5	489.7 ± 19.8	498.5 ± 19.2
Cover Stick	1.5	61.3 ± 3.5	50.3 ± 5.6	55.3 ± 1.8	63.4 ± 3.2	58.9 ± 2.9	56.4 ± 4.8
	3.0	123.2 ± 8.1	118.9 ± 6.5	120.3 ± 5.8	124.5 ± 6.1	120.9 ± 7.1	119.8 ± 6.3
After Sun Spray	1.5	289.7 ± 13.6	312.2 ± 12.6	305.1 ± 12.9	302.4 ± 21.4	320.8 ± 18.7	311.4 ± 9.9
	3.0	589.6 ± 21.4	611.2 ± 23.4	601.3 ± 5.9	598.5 ± 18.9	620.4 ± 15.6	609.8 ± 18.2

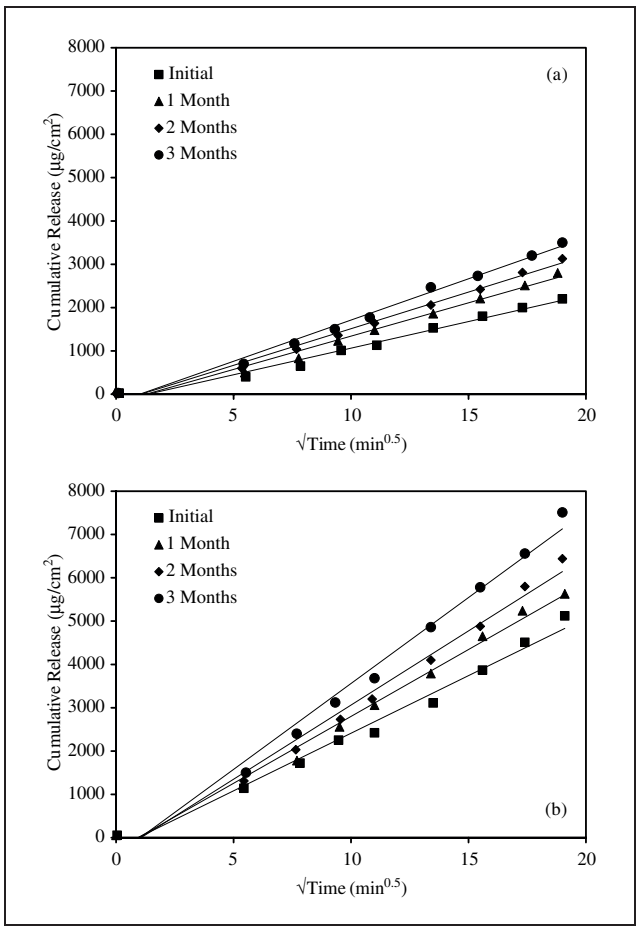

Fig. 2: Comparison of triclosan release from (a) 1.5% and (b) 3% cream using ethanol: water (80% v/v) as the dissolution medium. Results are for creams stored at 25 °C ± 60% RH for 3 months

2.3. Inhibition zone

Inhibition zone studies performed initially and after 3 months (Table 7) showed that the triclosan released from the topical vehicles did inhibit the growth of three microbes *Staphylococcus epidermidis, Streptococcus faecalis* and *Propionibacterium acnes*. However, increasing the triclosan concentration from 1.5 to 3% did not double the activity against the microbes. This meant the minimum inhibitory concentration was already reached at 1.5% triclosan. Although the inhibition of 3% was not higher than that of 1.5%, its duration of action may be higher. In all the products triclosan showed significantly higher activity against *Staphylococcus epidermidis* and *Propionibacterium acnes* but poor activity against *Streptococcus faecalis*. The results in Table 7 show that the microbicidal activity of triclosan did not decrease after storage for 3 months at low and high temperatures. The antimicrobial activity was highest in the foot gel and after sun spray containing 20–25% ethanol. Ethanol is an effective antimicrobial agent and combined with triclosan the results show the magnitude of the synergistic effect. The weakest activity was observed for the cover stick. These differences could be the result of differences in the release rate of triclosan from these products in addition to the effect of alcohol.

2.4. Correlation between release and microbicidal activity

The order for the release and antimicrobial activity, from highest to lowest, for the five products were: after sun spray > foot gel > emulsion > cream > cover stick for release into the ethanolic solution (Table 6) and after sun spray ≥ foot gel > emulsion ≥ cream > cover stick for zone inhibition (Table 7). This results suggest that there is a relationship between the rate of triclosan release from topical vehicles and the antimicrobial activity. To explore this apparent correlation between release and antimicrobial activity one batch of each of the products containing 3% triclosan and stored for 2 months at 25 °C were used to measure both the release and the effect on inhibition zone during release. Release was performed and the samples taken after 60, 120, 240 and 360 h were analyzed for triclosan content and then also used to measure the inhibition zone against *Staphylococcus epidermidis*. The inhibition zones of control samples consisting of the dissolution medium were also measured and subtracted from the results of test solutions to estimate the effect of triclosan.
In Fig. 3 the results from the correlation studies are shown. For each product the size of the inhibition zone was plotted against the concentration of triclosan released measured by HPLC. For all the products there was a linear relationship because the mean correlation coefficient was $R^2 = 0.97$ (min. = 0.92; max. = 0.99) as shown in Fig. 3. This means that an increase in the amount of triclosan release resulted in an in crease in inhibition zone according to the relationship [triclosan} α size inhibition

Table 7: Average inhibition zones (n = 3) measured over 3 days for three microorganisms

Formula	Conc. (%)	Zone Inhibition (mm)					
		Staphylococcus epidermidis		*Streptococcus faecalis*		*Propionibacterium acnes*	
		25 °C	40 °C	25 °C	40 °C	25 °C	40 °C
Cream	1.5	10.05	9.61	0.98	0.70	3.38	3.20
	3.0	10.47	9.46	1.31	1.01	3.84	3.78
Emulsion	1.5	8.68	8.42	0.70	0.73	2.94	3.56
	3.0	8.91	8.90	0.71	0.62	3.20	3.89
Foot Gel	1.5	13.80	18.32	7.72	4.86	16.97	15.53
	3.0	14.78	16.03	5.13	2.58	17.59	15.54
Cover Stick	1.5	8.51	6.56	0.73	0.58	2.31	2.67
	3.0	9.68	10.49	1.00	0.72	2.71	2.67
After Sun Spray	1.5	19.80	16.47	5.08	3.46	14.23	15.14
	3.0	15.30	14.26	4.68	4.15	14.26	16.75

Results are for the five topical products after 3 months storage at 25 and 40 °C

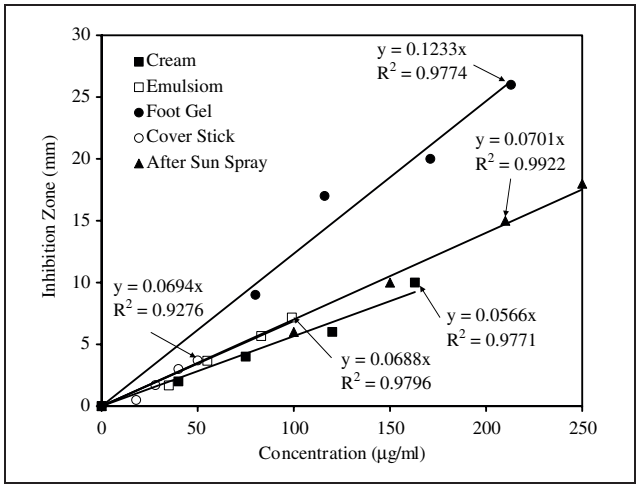

Fig. 3: Linear correlation between inhibition zone and concentration of triclosan release from topical formulations after 60, 120, 240 and 360 min. Results are for product containing 3% triclosan and stored for 2 months at 25 °C

zone. The results show that possibly *in vitro* release studies, in this case using Enhancer cells, can be used to predict the antimicrobial activity of triclosan in topical formulations during quality control. The advantages are that release studies are much cheaper and less time consuming than inhibition zone tests.

2.5. Conclusions

This study reports the formulation of five stable topical delivery systems containing triclosan. *In vitro* release studies showed that triclosan was released from these products in a predictable manner and that the released triclosan was effective against microbes commonly found on the skin. There was a linear relationship between the amount of triclosan released from the formulations and the microbicidal effect suggesting good *in vitro/in vivo* correlation. This means that for triclosan *in vitro* release studies can be used to predict its antimicrobial activity.

3. Experimental

3.1. Materials

Triclosan (Irgasan DP 300, Ciba Specialty Chemicals, Basel, Switzerland) was obtained from Adcock Ingram, Ltd. (Krugersdorp, South Africa). Arachis oil, polyoxyethylene sorbitan monooleate, sorbitan monooleate, methyl hydroxybenzoate, propyl hydroxybenzoate, sorbitol, liquid paraffin, propylene glycol, ethanol, citric acid, stearic acid, anhydrous monosodium phosphate, trisodium phosphate dodecahydrate, butylated hydroxyanisole, methanol and orthophosphoric acid was obtained from Saarchem Chemicals (Krugerdorp, South Africa). Polyoxyl castor oils (Cremophors), acid stable glyceryl monostearate, cetyl stearyl 2-ethyl hexanoate (Luvitrol EHO) and dexpanthenol was obtained from BASF (Midrand, South Africa). Amorphous colloidal silicon dioxide (Aerosil, Degussa Africa (Pty) Ltd., Midrand, South Africa) and a mixture of acid glycol esters, tribehenin and synthetic beeswaxes (Synchrowax, Croda Chemicals SA Pty Ltd, Kempton Park, South Africa) and PPG-2 myristyl ether propionate (Crodamol PMP, Croda) were also used.

3.2. Preparation of topical products

In this study, five skin formulations for the topical delivery of triclosan were developed. The formulae and methods of preparation for these vehicles are given in Tables 1 to 5. Skin cream, lotion, cover stick, foot gel and after sun spray are represented.

3.3. Chromatographic methods of analysis

A Hewlett Packard 1050 HPLC (Agilent, California, USA) equipped with a diode array UV detector, quaternary gradient pump, auto-sampler and Chemstation® revision A.06.02 control and data analysis software was used. The column was a Luna C_{18} (5 µm, 150 × 4.6 mm, Phenomenex, California, USA) and the mobile phase methanol: water (85 : 15 v/v) containing 0.1% ortho-phosphoric acid at a flow rate of 1 ml/min. The injection volume was 10 µl, with UV detection at 210 nm at ambient temperature. The retention time for triclosan was approximately 5.2 min. Standards were prepared by dissolving 20 mg of triclosan in 100 ml methanol, and diluting further to obtain standards of 25, 50, 100 150, 200 and 250 µg/ml. The same set of standards was used for assay and release testing.

The method was validated according to the ICH-Q2A guidelines (ICH 1995). Accuracy was determined by weighing the appropriate amount of cream placebo into 50 ml volumetric flasks and spiking by adding standard solutions obtain 3 samples each of 80%, 100% and 120% of the expected sample concentration. Precision was tested by analyzing 9 samples of the same homogenous cream on day one, and analyzing 3 samples again on two more days. Specificity was tested by using forced degradation. A standard solution of triclosan was diluted to 50% with water, 1 M hydrochloric acid, 1 M sodium hydroxide and 5% hydrogen peroxide, and stored at 40 °C overnight to degrade and then analyzed. This method with a mobile phase of methanol : water (60 : 40 v/v) containing 0.1% ortho-phosphoric acid at a flow rate of 1 ml/min and UV detection at 254 nm was used to assay the preservatives methyl and propyl paraben. The retention time for methyl paraben was 4 min and propyl paraben 9.5 min.

The concentration of alcohol in the foot gel and after sun spray was measured by gas chromatography. Samples of 1 µl were analyzed with a HP 6890 series GC (Agilent, California, USA). A Poropak Q (100 to 120 mesh, Markes International Limited, Pontyclun, United Kingdom) column at 150 °C with the injection and detector temperature set at 250 °C. Isopropyl alcohol was used as the internal standard.

3.4. Stability testing

Five products were formulated in this study. Each product was formulated containing either 1.5 or 3% triclosan. For each formula three batches was manufactured. In total 30 trial batches ($5 \times 2 \times 3 = 30$) were manufactured and tested. All trial batches were stores at 3 temperatures: 5 °C, 25 °C combined with 60% relative humidity, and 40 °C with 75% relative humidity. Stability tests were conducted initially and repeated after 1, 2 and 3 months. In additions to assay and *in vitro* release the following tests were also performed on the products at these time-points. Preservative efficacy was determined as described in the USP (USP XXIV 2000). Changes in pH was measured with a Mettler MP 220 pH meter (Mettler-Toledo, Columbus, OH, USA), relative density was measured with an Anton Paar DMA 38 density meter (Anton Paar, Hertford, United Kingdom) and viscosity was measured with a Brookfield DV-II viscometer (Brookfield Engineering, Middleboro, MA, USA). In addition the mass loss, spreadibility (Narsai et al. 1997) and particle size (Galai-Cis-1 particle size analyser, Galai, Ltd., Migdal Ha'Emek, Israel) were also determined. For the after sun spray formula changes in the size of the spray pattern and discharge mass were also determined.

3.5. In vitro release test

One batch of each of the five formulated products was chosen for the release studies. Batches containing 3% triclosan and stored a 25 °C were used. The release of the active ingredient, triclosan, was measured using Enhancer® cells using a modified USP six spindle dissolution test apparatus. The Enhancer cells used in this study consisted of a Teflon load ring, a cap, a membrane, and a drug reservoir. The semisolid preparation was placed in the drug reservoir (2 cm diameter) on top of the membrane. A metal load ring was used to keep the membrane or skin and the washer in place during the cap application. Finally, the bottom screw was tightened to bring the semisolid preparation, in complete contact with the membrane making certain that no entrapped air is present at the interface of the semisolid preparation, and the membrane. A USP method 2, paddle, dissolution tester (Vanderkamp 700, Van Kel Industries, NJ, USA), with modified flask assemblies consisting of 200 ml flasks instead of the standard 900 ml and smaller sized paddles, was used to measure drug release from the enhancer cell assembly. The membranes used in this study were cellulose acetate with a pore size of 0.45 µm soaked in 15% isopropyl myristate. A combination of ethanol : water (80 : 20 v/v) at 32 °C was used as dissolution medium and the rotation speed of the paddles was set at 100 rpm. Samples (1 ml) were withdrawn at intervals up to 360 min. These samples were analyzed using the described HPLC method to determine the amount of triclosan released versus time. Tests were done in triplicate.

3.6. Zone inhibition test

This test determined the ability of the triclosan preparations to inhibit the growth of *Staphylococcus epidermidis, Streptococcus faecalis* and *Propionibacterium acnes* (Grove et al. 2003). Sterile molten TS agar was prepared and allowed to cool to 45 °C. Whilst the agar was left to cool, 0.1 ml of solutions containing the respective micro-organisms were pipetted into sterile Petri dishes. The agar was poured into the petri-dishes

and swirled to mix the agar and the micro-organisms. The plates were left to cool and set and then it was incubated for 2 h at 37 °C. A cork bore was used to make holes in the agar in middle of each petri-dish. The bottom of the holes was sealed with molten agar to stop diffusion of the liquid test products underneath the agar. Approximately 0.1 ml of the test preparations was poured into the holes and then the plates were incubated for 48 h at 37 °C. This test measures the ability of triclosan solutions to diffuse into the agar and kill the micro-organisms. After incubation a Vernier calliper was used to measure the angular radius of the zone that formed around the hole. The inhibition zones of control solutions containing the dissolution medium were also determined and subtracted from the results of the sample inhibition zones. This was done to ensure that the inhibition obtained was from the triclosan released and not the dissolution medium. Tests were done in triplicate.

3.7. Statistical analysis

All calculations were performed in Microsoft Excel (Microsoft, Seattle, WA, USA). Multivariate analysis of variance (MANOVA), including a post hoc comparison using the Newman-Keuls test, was performed on the mean inhibition zones, release and stability values to identify significant differences in stability, release and antibacterial activity (Statitica 5.1, Statsoft Inc., Tulsa, OK, USA). P-values of less than 0.05 indicated significant differences.

Acknowledgement: This work was supported by a grant from the National Research Foundation of South Africa (Pretoria, South Africa).

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