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Comparison of free and bound iodine and iodide species as a function of the dilution of three commercial povidone–iodine formulations and their microbicidal activity

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Abstract

Equilibrium dialysis on povidone–iodine-solutions (Braunol®, standardized Betadine® and non-standardized *iso*-Betadine®) reveal that the amount of available iodine, free iodine, iodide and triiodide varies significantly both in the undiluted and diluted forms. These differences are reflected in the different bactericidal activity against *Staphyloccus aureus* as determined by the standard quantitative in vitro suspension test. The amount of available iodine is not an appropriate measure for an assessment of the microbicidal activity. For this, the free iodine has to be determined by means of equilibrium dialysis. The free iodine concentration in the Braunol® concentrate was found to be 22 mg/L, in the standardized Betadine® 9.7 mg/L and in the non-standardized Betadine® concentrate only 2.1 mg/L. Because of the atypical behaviour of iodophores and the increase of free iodine at dilution and because of a bactericidal level of free iodine of 5 mg/L, Braunol® and standardized Betadine® can be employed as disinfectant as such, *iso*-Betadine® has to be diluted before use. Summarizing all results, it can be stated that Braunol® is superior to standardized Betadine and unstandardized *iso*-Betadine® both as to the release of free iodine in the undiluted and in the diluted forms as in the killing rate of *S. aureus*.
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1. Introduction

It is well known from the detailed work of Horn and Ditter (1983), that binding of iodine in aqueous povidone–iodine (PI) solutions is complex, the free species of iodine is formally controlled by the mass action law including coupled reversible interactions between (a) iodine/iodide, (b) triiodide/polymer and (c) iodine/triiodide-polymer complex. The coupling of these equilibria constitutes in aqueous PI-concentrates a remarkable reservoir effect of free iodine, the measure for bactericidal potency.

It was the purpose of this work to compare three commercial aqueous PI solutions, *iso*-Betadine® and Braunol®, both available on the Belgian market, containing, respectively, 10% PI and 7.5% PI and Betadine®, available on the Swiss market. Braunol® and Betadine® are both stabilized with $NaIO_3$ while *iso*-Betadine® is not. Betadine® is thus henceforth referred to as standardized Betadine® while *iso*-Betadine® is referred to as unstandardized *iso*-Betadine®. The three commercial solutions were analyzed for their content of free and bound species of iodine, iodide and triiodide both in the non-diluted and in the diluted states using equilibrium dialysis. Their microbicidal activities were then compared.

2. Materials and methods

2.1. Test products

Braunol® (7.50 g PVP-I_2/100 mL solution) (B. Braun Medical, Melsungen, Germany) and standardized Betadine® (Mundipharma, Basel, Switzerland) and unstandardized *iso*-Betadine® (10 g PVP-I_2/100 mL solution) (Viatris, Brussels,

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Belgium) were used as such. Sodium thiosulfate, starch and KI were employed for the analysis of available iodine. All reactants were of analytical quality.

2.1.1. Micro-organisms

Test strains were standard ATCC strains, obtained from freeze-dried stock cultures: *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442. The bacterial suspension was obtained from a freshly grown culture on a TSA (Tryptone Soya Agar, Oxoid, Hampshire, England) and adjusted to a concentration of 10^9 colony-forming units per millilitre by turbidimetry. The inoculum size was determined by the surface culture technique.

2.2. Chemical analysis

Determination of the total available iodine: concentrations of total available iodine were determined according to the procedure as described in the USP (USP, 2004).

Determination of free iodine in non-diluted and diluted solutions: free iodine was determined with equilibrium dialysis at 25 °C by means of a Kontron Diapack system, a donor/acceptor volume ratio of 2:2, using polyethylene membranes (PE, high pressure PE, thickness 50 μm; Odenwald Chemie, Schönau, Germany) and after steady rotation for 19 h. The commercial solutions were diluted up to a concentration of 0.191 g/L PVP-I_2 for Braunol® and 0.25 g/L PVP-I_2 for standardized Betadine® and unstandardized *iso*-Betadine®, respectively. At equilibrium, the amount of free iodine was quantitatively transformed into I_3^- by the addition of an excessive amount of KI and spectrophotometrically determined at 352 nm.

Calibration line of I_2: a stock solution of 100.0 mg I_2 and 1.5 g KI was dissolved in water (milli-Q-water) and adjusted to 100 mL with the same solvent. For the calibration line, dilutions of the stock solution were made with freshly prepared KI solutions (5.00 g KI in 100 mL of water).

Determination of free iodide: after equilibrium dialysis at 25 °C, using a hydrated cellulose membrane (Mwt cut off 3500) (Spectrum Laboratories, CA, USA) and a donor acceptor volume ratio of 20:2, 1 mL free iodide was taken from the acceptor compartment, diluted to 5 mL with water and the concentration determined at a wavelength of 226 nm (specific I^- band) using a calibration curve of KI.

Calibration line of KI: from a freshly prepared KI stock solution (0.0069 g/100 mL water) dilutions were made, taking 1–20 mL and diluting to 100 mL with water.

Determination of free triiodide: the concentration of free I_3^- was evaluated by means of the triiodide equilibrium at 25 °C (Horn and Ditter, 1983).

$$\frac{(I_3^-)}{(I_2)(I^-)} = K_1, \quad K_1 = 770\,M^{-1} \tag{1}$$

In view of the extremely small values of the equilibrium constants K_2 and K_3 of IO^- and IO_3^-:

$$\frac{(I^-)(H^+)(HIO)}{(I_2)} = K_2, \quad K_2 = 2.58 \times 10^{-13}\,M^2 \tag{2}$$

$$\frac{(I^-)^5(H^+)^6(IO_3^-)}{(I_2)^3} = K_3, \quad K_3 = 2.7 \times 10^{-48}\,M^9 \tag{3}$$

possible present in the solutions, these species are negligible and are not considered in the analysis (Allen and Keefer, 1955; Awtrey and Connick, 1951; Chang, 1958). Similarly, complexes involving I_2 and I_3^-, such as I_5^-, I_7^-, etc., are present in the free state only in minor concentrations and are also neglected (Pearce and Eversole, 1924).

Finally, the bound fractions of the different species were calculated from the mass balance, taking into account the concentration of PI.

2.3. Quantitative in vitro suspension test

The bactericidal activity of the three commercial aqueous PI solutions was determined by using the standard quantitative in vitro suspension test as proposed by the European Committee for Standardization EN 1040. All tests were carried out in a water bath maintained at 20 ± 1 °C and all reagents were equilibrated for at least 30 min at this temperature.

2.3.1. Preparation of the bacterial suspension

Freeze-dried stock cultures were used. Continuous cultures were maintained on tryptic soy agar (TSA) slants by daily transfers and incubation at 37 °C. Tests were made between the 3rd and the 14th subcultures. A TSA slope from a freshly grown culture was inoculated and incubated at 37 °C. After 24 h, the growth was washed out in diluent (Sodium chloride 8.5 g, tryptone 1 g in 1000 mL distilled water) using glass beads and filtered through glass–wool. The bacterial suspension was standardized turbidimetrically to give a concentration of approximately 10^9 colony-forming units per millilitre and the inoculum size was determined by the surface culture technique.

A 0.1 mL bacterial suspension was added to 10 mL disinfectant and left for, respectively, 15, 30 and 60 s. Subsequently a 1 mL aliquot of this mixture was neutralized with 9 mL inhibitor LPHT (lecithin 0.3%, polysorbate-80 3%, histidine 0.1%, sodium thiosulfate 0.5%) for 5 min, preventing carry-over of the disinfectant. For each product, the neutralizer was validated.

The count of survivors in the neutralized mixture was performed by the surface culture technique. One millilitre and 0.1 mL of the resulting mixture was spread on TSA agar medium distributed in Petridishes and incubated for 24 h at 37 °C.

The control procedure was carried out by using sterile distilled water instead of the disinfectant. Each assay was performed three times and in water of standardized hardness.

The germicidal effect (GE) of the test product was calculated and expressed as logarithm of the number of colony-forming units per millilitre in the test mixture without disinfectant (N_C) minus the number of colony-forming units after the contact with the disinfectant (N_D) ($GE = \log_{10} N_C - \log_{10} N_D$).

3. Results

3.1. Determination of the total available iodine

Following the procedure of the USP (USP, 2004), an amount of 0.78 g available iodine/100 mL was found for the Braunol® solution, 0.97 g/100 mL for standardized Betadine® and 0.97 g/100 mL for the unstandardized *iso*-Betadine® solutions, respectively.

3.2. Determination of free iodine in the non-diluted solutions

To allow passage of I_2 vapour, from donor to acceptor compartment during the dialysis process, polyethylene membranes were used. Equilibrium dialysis was attained under constant rotation after 19 h. The free iodine concentration in the Braunol® concentrate was found to be 22.0 mg/L, in the standardized Betadine® concentrate 9.7 mg/L and only 2.1 mg/L in the unstandardized one, despite a higher total amount of available iodine in both Betadine® solutions in comparison to Braunol®. In all three concentrates, only about 0.3, 0.09 and 0.02% of available iodine is present as free iodine for Braunol®, standardized Betadine® and unstandardized *iso*-Betadine®, respectively. The rest is present in complex form constituting an iodine reservoir. The microbicidal concentration of free iodine is at least 5 mg/L (Horn et al., 1987). This amount is not present in the concentrate of unstandardized *iso*-Betadine® but is noted in the concentrate of Braunol® and the standardized Betadine® solutions.

The results of the three PI solutions, after dilution, are shown in Fig. 1. From the figure it can be noted that the amount of free iodine is higher in the Braunol® solution up to a dilution factor of 100.

The figure illustrates that, on dilution of the concentrate, the concentration of free iodine increases up to a maximum of about 1.53 g/L povidone–iodine content after a 50-fold dilution for Braunol® and of about 1–2 g/L povidone–iodine (a 50–100-fold dilution) in both Betadine® solutions.

For Braunol® a maximum of 51 mg/L free iodine is found. For the other two solutions, the maxima of free iodine are 35 and 31 mg/L, respectively, for the standardized and unstandardized one.

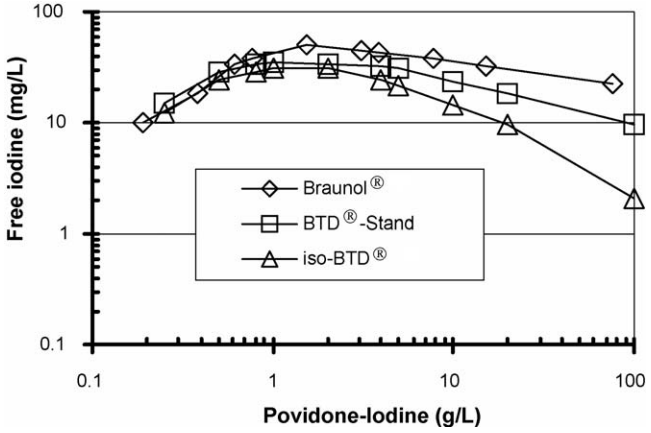

Fig. 1. Free iodine as a function of the concentration of povidone–iodine.

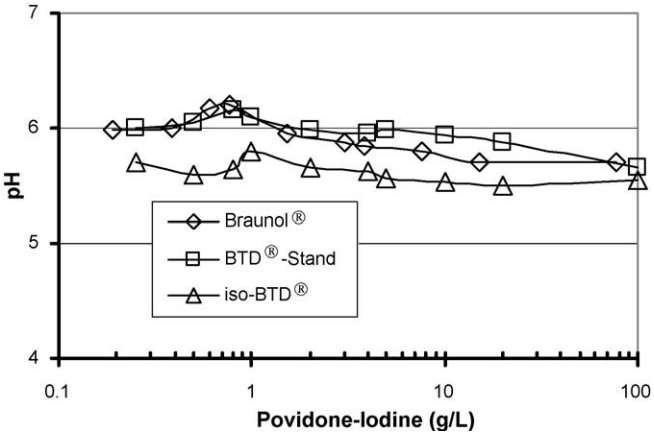

Fig. 2. pH as a function of the concentration of povidone–iodine.

The key to understand this conspicuous feature of PI solutions is found in specific interaction tendencies towards PVP of the iodine and iodide constituents (see further).

3.3. pH-values of diluted solutions

The pH of the diluted solutions was recorded and presented in Fig. 2.

As it is seen from the figure, the pH-values are increasing as a function of the dilution, starting from pH 5.55 up to 5.80 for unstandardized *iso*-Betadine®. The pH-values for the Braunol® and standardized Betadine® dilutions are higher, starting from 5.65 up to 6.20. These values remain virtually constant with increase in dilution factors.

3.4. Determination of free iodide and calculation of free triiodide and complexed triiodide

When using the same volume in donor and acceptor cells, a high osmotic interference was noted, as seen in Fig. 3. To avoid these interferences, a donor and acceptor volume ratio of 20:2 was employed. Equilibrium was attained under constant overnight rotation.

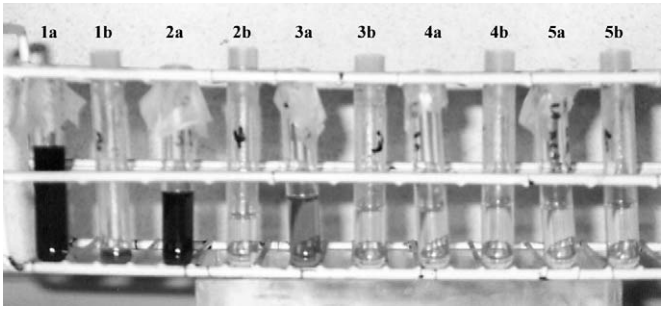

Fig. 3. Influence of osmotic pressure on equilibrium dialysis (a, donor compartment; b, acceptor compartment; 1, non-diluted solution; 2, 20 g PVP-iodine/L; 3, 5 g PVP-iodine/L; 4, 1 g PVP-iodine/L; 5, 0.25 g PVP-iodine/L).

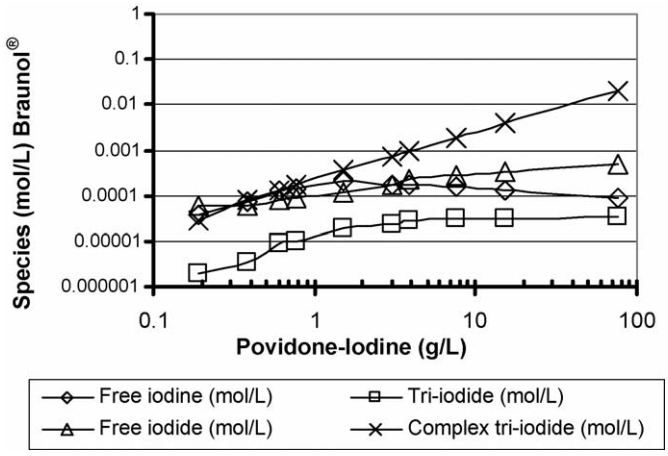

Fig. 4. Concentration of different species as a function of Braunol® dilution.

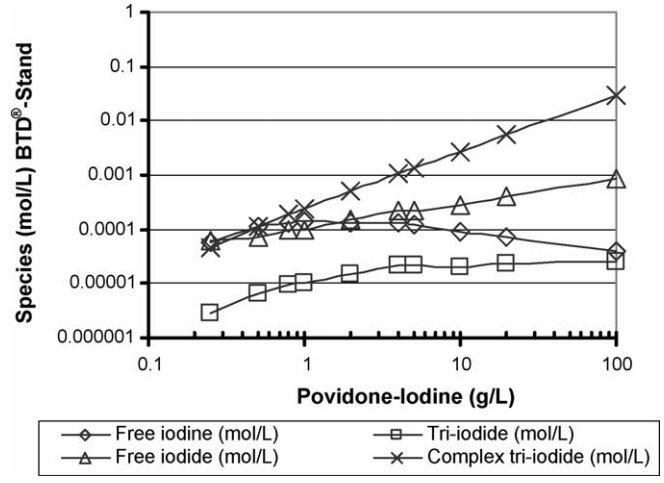

Fig. 5. Concentration of different species as a function of standardized Betadine® dilution.

In Figs. 4–6, the experimental results from equilibrium dialysis with regards to the free species I_2 and I^- and the calculated species I_3^- and PI_3^- (the formal composition of the polymer–iodine–iodide complex) are plotted as a function of

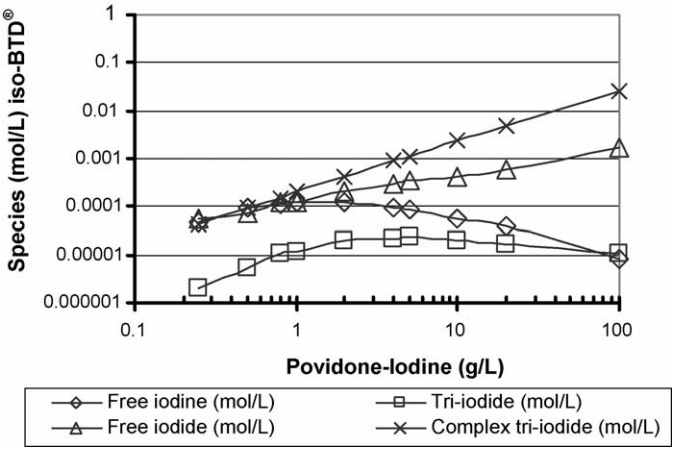

Fig. 6. Concentration of different species as a function of unstandardized *iso*-Betadine® dilution.

Table 1
Bactericidal effect of three non-diluted povidone–iodine compounds

Test product	Log_{10} reduction factors at specified time (s)[a]					
	S. aureus			*P. aeruginosa*		
	15	30	60	15	30	60
Braunol®	>5	>5	>5	>5	>5	>5
	>5	>5	>5	>5	>5	>5
	>5	>5	>5	>5	>5	>5
Unstandardized *iso*-Betadine®	0.63	1.10	4.43	>5	>5	>5
	0.91	1.41	4.04	>5	>5	>5
	1.17	1.26	2.91	>5	>5	>5
Standardized Betadine®	1.28	3.78	>5	>5	>5	>5
	1.51	4.80	>5	>5	>5	>5
	1.82	3.83	>5	>5	>5	>5

[a] Each assay was performed three times.

the concentration of povidone–iodine. For the three preparations, the same pattern is observed.

While the concentrations of free I^-, free I_3^- and PI_3^- are steadily increasing with increasing povidone–iodine content, the concentration of free iodine, I_2, passes through the already mentioned maximum of about 51, 35 and 31 mg/L, respectively, for Braunol®, standardized Betadine® and unstandardized *iso*-Betadine®. At these concentration levels, between 55 and 57% of the available titratable iodine is present in a complexed state. At very low concentrations of povidone–iodine, i.e. less than 0.38 g/L for Braunol®, and less than 0.5 g/L for standardized Betadine® and less than 0.8 g/L for *iso*-Betadine® unstandardized, the polymer iodine complex is very low ($<10^{-4}$ M).

3.5. Microbicidal effect of the PI compounds

The results of the microbicidal activity of the products are summarized in Table 1.

4. Discussion

Despite a higher total amount of available iodine in both Betadine® solutions than the Braunol® solution, the free iodine concentration was higher in Braunol® (22.0 mg/L) than the other two (9.7 mg/L for standardized Betadine® and 2.1 mg/L in unstandardized *iso*-Betadine®, respectively).

The microbicidal concentration of free iodine is at least 5 mg/L (Horn et al., 1987). This amount is not present in the concentrate of unstandardized *iso*-Betadine® but is noted in the concentrate of Braunol® and the standardized Betadine® solutions.

The results of the three PI solutions, after dilution show that the highest amount of free iodine is attained in Braunol® (51 mg/L) with the maxima for standardized Betadine® being 35 and 31 mg/L for *iso*-Betadine®, respectively. Hence, the concentration of free iodine as a function of the dilution to the maximum of free iodine approximately fluctuates within a factor of ca. 2, 3.6 and 15 for Braunol®, standardized Betadine® and unstandardized *iso*-Betadine®. This fairly constant level of

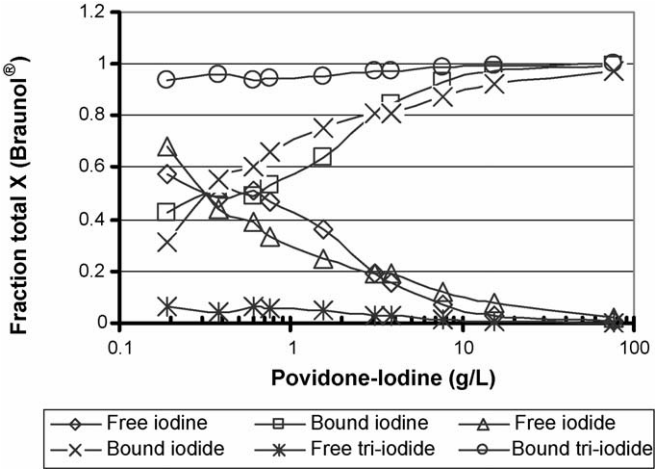

Fig. 7. Fraction of total X as a function of Braunol® dilution.

free iodine in the Braunol® and standardized Betadine® solutions is attributed to the molar ratio of I_2/I^-, which is maintained between 1 and 2, by lowering the I^- content and by the addition of iodate in the formulation.

The change in pH in all the solutions is much less than with non-buffered solutions; indeed, Horn and Ditter found, with non-buffered solutions, an increase of pH from 2 up to 5 (Horn and Ditter, 1983). The pH > 5, in the Braunol® and standardized Betadine® solutions is necessary for controlling the Dushman reaction (Dushman, 1904; Horn et al., 1987), which would raise the free iodine concentration to levels, that could be intolerable to the skin (Gottardi, 1991). The observed pH for all three solutions is advantageous since their values approximate the pH of the skin at any dilution.

The unusual concentration dependence of the free species, I_2, I^- and I_3^- on total I_2 content can be described by the coupling of the triiodide equilibrium (Eq. (1)) with two independent equilibria:

$$\frac{(PI_3^-)}{(I_3^-)(P)} = K_4, \quad K_4 = 310\,\mathrm{M}^{-1} \tag{4}$$

$$\frac{(PI_5^-)}{(PI_3^-)(I_2)} = K_5, \quad K_5 = 6.4 \times 10^4\,\mathrm{M}^{-1} \tag{5}$$

where P denotes the concentration of the monomer structural units of the polymer and with PI_3^-, PI_5^-, PI_7^-, ... with overlapping domains of existence (Horn and Ditter, 1983).

The binding pattern of different species towards PVP can best be deduced from the graphs presented in Figs. 7–9, where the concentrations of the free and the bound fractions of the constituents are compared. From all constituents present in the system, at the higher PI concentrations, I_2 and iodide to a lesser extent is preferentially bound to PVP. Within the lower concentration range, nearly half of the iodine and iodide remain in a free state. This observation is valid for all three solutions.

The marked reservoir effect observed for iodine in PVP-I_2 solutions constitutes the basic difference to the concentration behaviour of aqueous Lugol's solutions, in which iodide is added for increasing the solubility of iodine.

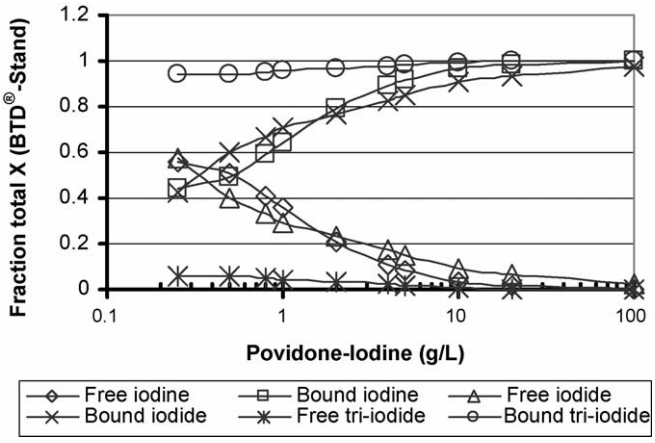

Fig. 8. Fraction of total X as a function of standardized Betadine® dilution.

As it was demonstrated in the work of Horn and Ditter (1983), the free iodine concentration in a Lugol's solution exceeded that of the used PI solution by two orders of magnitude. Gottardi (1991) notes that in the Lugol's solution 170 mg/L of free iodine is present, this is 85, 17 and 8.5 times more concentrated than in the *iso*-Betadine® non-standardized, standardized Betadine® and Braunol® solutions, respectively. The well-established microbicidal activity of Lugol's solution is limited in its clinical use, due to the presence of this high free iodine concentration, leading to an intensively irritating effect to abraded tissue and mucous membranes. Povidone–iodine, on the other hand, is non-irritating and exhibits nevertheless the microbicidal properties of iodine (Trueman, 1971).

The plots of free iodine as a function of dilution do not follow the normal behaviour of complexes in aqueous solutions and in this way influence the antimicrobial properties against e.g. *S. aureus*. This is attributed to the complex behaviour of iodine in the presence of povidone, iodide, the existing ratio of I_2/I^- and the addition of stabilizing species (iodate). The undiluted solutions show a remarkable reservoir of iodine. The bactericidal level of at least 5 mg/L free iodine is found in two of the three PI solutions. The main difference between the formulations is that the 7.5% PI solution (Braunol®) and 10% PI solution (standardized Betadine®) are products of a second generation, in

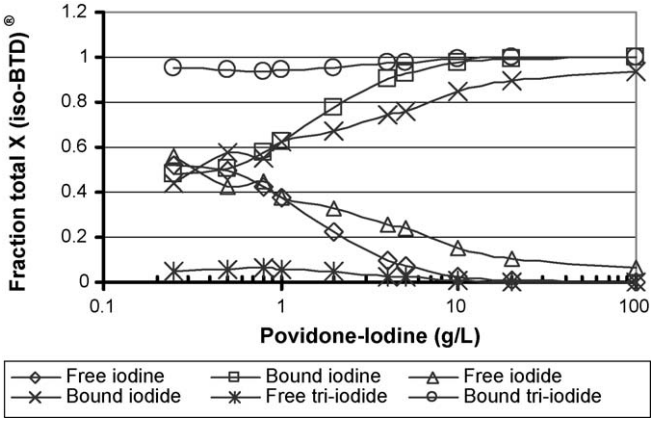

Fig. 9. Fraction of total X as a function of unstandardized *iso*-Betadine® dilution.

which the liberation of free iodine is physico-chemically controlled. This explains why the concentration of free iodine in these preparations is better standardized than in the unstandardized *iso*-Betadine® and thus fluctuates less. These differences therefore explain the differences in the killing rates and the microbicidal activity.

The antimicrobial effect against *S. aureus* varied markedly between the three products and for the different contact times (Table 1). The Braunol® solution reached a reduction factor of more than 5 for all three contact times. Non-standardized *iso*-Betadine® showed a mean reduction factor of 0.9 after 15 s, 1.2 after 30 s and of 3.8 after 60 s. A bactericidal effect of 99.999% could not be demonstrated. With standardized Betadine®, a $\log_{10}$ reduction factor of 1.5 was obtained after 15 s, and of 4.1 after 30 s. After 60 s, a $\log_{10}$ reduction factor of more than 5 was obtained.

On the other hand a $\log_{10}$ reduction factor of more than 5 was obtained in 30 s for both bacterial strains with a 10% diluted solution of the three PI compounds. This also indicates that higher concentrations of free iodine are achieved in dilution series.

Despite the potential increase in killing rate after dilution, the risk of depleting the reservoir of available iodine, e.g. by interfering reactions of iodine with organic load e.g. blood, is also rapidly increasing. It was also demonstrated that the reversible maintenance of an effective level of free iodine over prolonged periods of exposure, can best be guaranteed with highly concentrated PI solutions.

Thus, Braunol® and standardized Betadine® can be used as such, while unstandardized *iso*-Betadine® has to be diluted before use in order to obtain an optimal bactericidal activity.

5. Conclusion

Three commercial PI solutions with different concentrations of available iodine were comparatively investigated for their free iodine levels in the non-diluted and diluted states. The amount of available iodine, as determined according to the USP (USP, 2004), is not a direct measure for free iodine in solution, the species that accounts for bactericidal activity. For that purpose, an analysis using equilibrium dialysis is necessary.

From the results above, the following conclusions can be made: the Braunol® and standardized Betadine® concentrate can be used undiluted, whereas the non-standardized *iso*-Betadine® solution has to be diluted before use.

The following chemical mechanism is valid for the three solutions: during the occurrence of bactericidal activity and other iodine consuming reactions, free iodine is reversibly delivered from the complexed state, according to a coupled system of complex reactions. Owing to this reservoir effect of aqueous PI concentrates, moderate, non-irritating, but however microbicidal effective concentrations of free iodine can be maintained at high levels of available iodine for prolonged periods of exposure, both for the Braunol® and standardized Betadine® as such, and for *iso*-Betadine® after dilution.

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