Relationship between Virucidal Efficacy and Free Iodine Concentration of Povidone-Iodine in Buffer Solution

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Povidone-iodine solutions prepared to various concentrations (0.01, 0.1, 1 and 10%) with 0.2M phosphate buffer (pH 7.0) (PVP-I PB) were analyzed to determine their free iodine concentrations using membrane permeation cells, and their inactivation effects on three viruses (influenza A virus, poliovirus type 1 and adenovirus type 3) were examined. The free iodine concentrations in the 0.01-10% PVP-I PB were determined to be 1.84, 4.88, 1.58 and 0.17 ppm (approximate values), respectively, with the maximum obtained for the 0.1% solution. The virucidal efficacy of these PVP-I PB against poliovirus type 1 and adenovirus type 3 was found to be generally dependent on free iodine concentration, with the 0.1% solution being the most effective. Influenza A virus was inactivated with an action time of 15 s at all four concentrations examined. The results of this study suggested an association between free iodine concentration and virucidal efficacy for the 0.01-10% PVP-I PB.

Key words : Povidone-iodine / Free iodine concentration / Virucidal effi- ciency / Permeation cell.

INTRODUCTION

Aqueous solutions of povidone-iodine (polyvinylpyrrolidone-iodine, hereinafter called PVP-I), a conjugate of polyvinylpyrrolidone with iodine, are commonly used as antiseptics. Iodine in PVP-I maintains an equilibrium in a wide variety of forms (e.g., I₂, I⁻, I³⁻, PVP-I₃⁻) in solutions, with the total amount of all these forms measured as available iodine content in sodium thiosulfate titration. Above all, free iodine (I₂), which is released by PVP-I, is reported to contribute to the biocidal activity of PVP-I, with the I₂ concentration increasing with increasing dilution rate for 10% PVP-I solution, and maximizing with a dilution rate of nearly 100 fold (Gottardi, 1983). With regard to PVP-I formulations, antibacterial effects (Atemnkeng et al., 2006; Berkelman and Holland, 1982), virucidal efficacy (Kawana, 1997; Sauerbrei and Wutzler, 2010) and free iodine concentrations in PVP-I (Atemnkeng et al., 2006; Gottardi, 1983; Horn and Ditter, 1983; Pollack and Iny, 1985) have been reported; however, few studies evaluate the association between virucidal efficacy and free iodine concentration. In addition, commercially available PVP-I formulations contain multiple additives, mainly surfactants, that are diverse in terms of type, quantity and pH. It is noteworthy that some surfactants contained in such commercial formulations have been reported to exhibit potent cytotoxicity (Iwasawa and Nakamura, 2001; 2003). Assessment of free iodine concentrations and biocidal activity, seemed to indicate that some of the wide varieties of additives contained in these liquid formulations might influence the equilibrium of the iodine species in the PVP-I PB to change the free iodine
concentrations, or have secondary effects (e.g., increase in permeability by surfactant) on the susceptibility of test microorganisms to iodine. Such an influence from additives could make it difficult to assess the association between free iodine concentration and biocidal effect. In addition, while the pH of a PVP-I solution shifts to the weakly acidic-neutral side when the solution is diluted with purified water, the 10% PVP-I solution exhibits strong acidity (pH: approx. 1.7), suggesting to be difficult in assessment of the association between free iodine concentration and biocidal effect due to the pH change at particular PVP-I concentrations. With this in mind, optionally chosen different concentrations of PVP-I PB were prepared using a pH 7 PB to avoid the influence of changes in pH and additive concentrations due to water dilution, and then assayed to clarify the relationship between the free iodine concentration and virucidal efficacy.

MATERIALS AND METHODS

Test solutions

PVP-I (PVP-Iodine, BASF Japan Ltd.) was dissolved in PB (adjusted to a pH of 7 by mixing 0.2M disodium hydrogen phosphate 12-hydrate, 7.16 g/100 mL, and 0.2M sodium dihydrogen phosphate anhydrate, 2.4 g/100 mL) to yield a 10% PVP-I PB (10 g PVP-I/100 mL), which was serially diluted 10 fold with PB to yield 1%, 0.1% and 0.01% solutions. The measured available iodine concentration of the 10% PVP-I PB was verified by titration with sodium thiosulfate.

Relationship between free iodine and absorbance

(Takikawa et al., 1978)

Iodine was accurately weighed and dissolved in a 10% potassium iodine (KI) aqueous solution to yield a solution having an iodine concentration of approximately 900 ppm, and then it was diluted with a KI solution to five iodine concentrations between 1.4 and 6.8 ppm. With the KI solution (diluent for the dilutions) used for a blank determination, the absorbance at 351.5 nm (A_{351.5}) was determined using a 1 cm cuvette, and the relationship between iodine concentration and absorbance was examined.

Measurement of free iodine concentrations in various test solutions

(Atemnkeng et al., 2006; Takikawa et al., 1978)

A high-density polyethylene membrane (DuraSeal, DIVERSIFIED BIOTECH) 0.04 mm in thickness was placed between two side-by-side membrane permeation cells for flat membranes (Permcell, VIDREX, KH-55, aperture dia.: 25 mm, membrane area: approx. 4.9 cm², inside volume: approx. 55 mL) (Fig.1), and a PVP-I PB was poured into the donor cell, and a KI solution into the acceptor cell. After stirring cell with a multi-stirrer at 25°C for 20 h, A_{351.5} of the KI aqueous solution in the acceptor cell was determined using a 1 cm cuvette, and the free iodine concentrations (ppm) in the various test solutions were determined using a regression line generated with the aforementioned iodine concentrations and absorbance values.

Preparation of test viruses

Influenza A virus (A/PR/8/34, H1N1, ATCC VR-95) was inoculated into the allantoic cavities of embryonated chicken eggs, which were incubated at 37°C. After 2-day incubation, the virus multiplying in the allantoic fluid were harvested, and concentrated using a hollow fiber cartridge (GE Healthcare); the concentrate was subjected to sucrose density gradient centrifugation (108,000 x g for 3 h at 4°C) to purify the virus, which was used as the test virus (range of viral concentrations in log_{10} TCID_{50} per mL: 8.7-9.5). For viral infectivity determination, Madin-Darby canine kidney (MDCK) epithelial cells were used.

Poliovirus type 1 (Poliovirus, strain sabin1 LSc 2ab, Japan Poliomyelitis Research Institute) was inoculated on vero cells. The virus-infected cells were incubated at 37°C for 2-3 d. When approximately 90% of the cells showed CPE, a cell lysate was prepared by freezing and thawing. The cell lysate was centrifuged at 2,380 xg for 10 min at 4°C, and the harvested supernatant was concentrated using an ultrafiltration membrane. The concentrate was then subjected to sucrose density gradient centrifugation (108,000 x g for 3 h at 4°C) to purify the virus, which was used as the test virus (range of viral concentrations in log_{10} TCID_{50} per mL: 8.6-8.9). For viral infectivity determination, vero cells were used.

Adenovirus type 3 (ATCC VR-3®) was inoculated on...
A549 cells. The virus-infected cells were incubated at 37°C for 2-3 d. When approximately 90% of the cells showed CPE, a cell lysate was prepared by freezing and thawing. The cell lysate was centrifuged at 2,380x g for 10 min at 4°C, and the harvested supernatant was concentrated using an ultrafiltration membrane. The concentrate was then subjected to sucrose density gradient centrifugation (108,000 xg for 3 h at 4°C) to purify the virus, which was used as the test virus (range of viral concentrations in log_{10} TCID_{50} per mL: 8.6-9.3). For viral infectivity determination, A549 cells were used.

**Subculture of the cells**

The cells were cultured using Dulbecco’s Modified Earle’s medium (DMEM, SIGMA Aldrich), supplemented with 5-10% fetal bovine serum (FBS, SIGMA Aldrich), with passage performed every 3-4 d in a CO2 incubator kept at 37°C to obtain subcultured cells, which were used in the following steps.

**Preparation of test solutions**

Four concentrations (0.01, 0.1, 1 and 10%) of PVP-I PB solutions, prepared in the same manner as for the measurement of free iodine concentrations, were used as the test solutions.

**Virus inactivation test**

All of the test procedures were implemented in an indoor environment at 25°C. An aliquot of 0.1 mL of each test virus suspension was added to 0.9 mL of the test solution, and the mixture was stirred by voltexing for 5 s to initiate the exposure. After a given exposure time, a 0.1 mL sample of the mixture was collected and diluted 5 fold with a 1.6-time concentration of Dulbecco’s PBS (Nissui Pharmaceutical Co., Ltd.) containing 0.5% sodium thiosulfate to stop the exposure of the test solution on the virus. This dilution was further diluted 100 fold with Dulbecco’s PBS to avoid the toxicity of sodium thiosulfate on the cells. This solution was used as the sample stock solution for viral infectivity determination. Baseline infectivity titer was established by inoculating 0.2M PB with each virus, immediately collecting a sample, and assaying the sample in the same manner.

**Measurement of viral infectivity**

The sample stock solution for viral infectivity determination was serially diluted with PBS 10 fold, after which 50 µL of the sample stock solution for infectivity titration or diluted virus and 50 µL of cells for viral infectivity determination in suspension in 5% FBS-supplemented DMEM were inoculated onto a 96-well microplate. Thereafter, the influenza virus and poliovirus were cultured in a 37°C CO2 incubator for 4 d. The adenovirus was cultured at 37°C for 3 d, after which the medium was replaced with 0.2% FBS-supplemented DMEM, and the virus was further cultured for 3 d. After completion of the cultivation, the CPE due to viral proliferation was examined under an inverted microscope, and viral infectivity (TCID_{50}/mL) was determined using the Reed-Muench method (Reed and Muench, 1938).

Log reduction values (LRVs) of viral infectivity were calculated using the equation: LRV = log_{10}(y) (baseline viral infectivity ÷ viral infectivity for each exposure time)

**RESULTS**

**pH of the test solutions**

The 10% PVP-I PB solution showed an available iodine concentration of 1.02% and a pH of 6.7. This solution was serially diluted 10 fold with PB to yield 0.01-1% solutions, all of which were found to have a pH of 7.0.

**Relationship between iodine concentration and absorbance at wavelength of 351.5 nm (A_{351.5})**

The relationship between iodine concentration and A_{351.5} is shown in Fig.2.

Values of A_{351.5} for five iodine concentrations (a: 1.4, b: 2.7, c: 4.1, d: 5.5, e: 6.8 ppm) were revealed a positive correlation between iodine concentration and A_{351.5}, with a regression line of Y = 0.1073X + 0.0153 and a coefficient of determination of R^2 = 0.9997.

![Fig. 2. Relationship between iodine concentration and absorbance at wavelength of 351.5 nm (A_{351.5}).](image)
Measurement of free iodine concentrations in the various test solutions

Mean free iodine concentrations from three measurements are plotted in Fig. 3.

Of the various test solutions, the 10% PVP-I PB solution was found to have a free iodine concentration outside the regression line range (approximate calculation from the regression line formula: 0.17 ppm); however, its 10-, 100- and 1000-fold dilutions were found to have free iodine concentrations of 1.58, 4.88 and 1.84 ppm, respectively, with the maximum obtained from the 100-fold dilution.

Virus inactivation test

The LRVs for the three viruses are shown in Tables 1, 2 and 3.

When the 0.01-10% PVP-I PB solutions were allowed to act on influenza A virus for 15 s, an infectivity titer reduction of ≥4 log was observed at all concentrations (Table 1).

When the PVP-I PB solutions were allowed to act on poliovirus, the exposure time to reach an LRV of ≥4 was determined to be 30 min for the 0.01% solution, 15 min for the 0.1% solution and 30 min for the 1% solution. With the 10% solution, even when allowed to act for 60 min, the LRV did not exceed 4 (Table 2). The virucidal efficacy (in terms of time to reach an LRV of ≥4; if there was no time difference, the virucidal efficacy was compared in terms of LRV) of the PVP-I PB solutions changed depending on the iodine concentration as follows: 0.1% > 0.01% ≥ 1% > 10%.

When the PVP-I PB solutions were allowed to act on adenovirus, the exposure time to reach an LRV of ≥4 was determined to be 5 min for the 0.01% solution, 1 min for the 0.1% solution, 5 min for the 1% solution and 60 min for the 10% solution (Table 3).

The virucidal efficacy of the PVP-I PB solutions changed depending on the iodine concentration as follows: 0.1% > 0.01% ≥ 1% > 10%.

DISCUSSION

In the present study, free iodine concentrations in PVP-I PB solutions and virucidal efficacy were examined under controlled conditions (as suggested from a preliminary study with 10% PVP-I aqueous solution) with the influence of pH avoided by dissolving PVP-I with PB, and likely changes in PB concentration (0.2M) due to dilution were suppressed by using a PB solution as the diluent.

There are three reported methods for measuring free iodine: extraction using the apolar solvent heptane (Pollack and Iny, 1985), equilibrium dialysis (Atemnkeng et al., 2006; Horn and Ditter, 1983) and potentiometry (Gottardi, 1983). In the present study, equilibrium dialysis was used in accordance with reported methods (Atemnkeng et al., 2006; Takikawa et al., 1978). The

![FIG. 3. Free iodine concentrations in 0.01-10% PVP-I PB solutions (n=3, mean). The vertical bars on the ⋄ marks indicate the standard deviations of three measured values. The iodine concentration was determined to be 1.84 ppm in the 0.01% solution, 4.88 ppm in the 0.1% solution, 1.58 ppm in the 1% solution and 0.17 ppm in the 10% solution (the 0.17 ppm value for the 10% solution was an approximation; the actual value was outside the regression line range).](image)

| Test drug       | Treatment time (s) | 15  | 30  | 60  |
|-----------------|--------------------|-----|-----|-----|
| PVP-I 0.01%     | >4.3               | n.d. | n.d.|
| PVP-I 0.1%      | >4.3               | n.d. | n.d.|
| PVP-I 1%        | 4.5±0.3            | n.d. | n.d.|
| PVP-I 10%       | 4.4±0.4            | 4.3±0.4| 4.4±0.5|
| Negative control| n.d.               | n.d. | -0.1±0.3|

| Test drug       | Treatment time (s) | 15  | 30  | 60  |
|-----------------|--------------------|-----|-----|-----|
| PVP-I 0.01%     | >4.3               | n.d. | n.d.|
| PVP-I 0.1%      | >4.3               | n.d. | n.d.|
| PVP-I 1%        | 4.5±0.3            | n.d. | n.d.|
| PVP-I 10%       | 4.4±0.4            | 4.3±0.4| 4.4±0.5|
| Negative control| n.d.               | n.d. | -0.1±0.3|

a: LRV = log_{10} (baseline viral infectivity/viral infectivity obtained with each exposure time)
The LRVs were calculated from viral infectivity titers determined from 0.01-10% PVP-I PB solutions with various exposure times.

Each test consisted of two to five measurements, and each LRV value is expressed as the mean of the repeated measurements. When all measurements are lower than the limit of detection, the LRV value is indicated as “>mean LRV.” For all other points, each value is expressed as the mean± standard deviation. As the negative control, only the infectivity titer following the longest exposure time with PB was measured.

b: The mean of two measurements was calculated.
c: The mean of five measurements was calculated.
d: Not determined.
membrane permeation system used was similar to a common side-by-side membrane permeation cells for flat membranes (Takikawa et al., 1978; Noda et al., 2009). Free iodine concentrations were measured using the same apparatus and a silicone membrane as in previously reported studies (Takikawa et al., 1978; Noda et al., 2009). However, both failed to assess free iodine concentrations with varied iodine or PVP-I concentrations, although some of the particular iodine or PVP-I concentrations were measured; therefore, their data did not serve for our objective. On the other hand, Atemnkeng et al. assessed free iodine concentrations in 10% solutions of two PVP-I formulations and their dilutions, using a special membrane permeation apparatus (Kontron Diapack system) and a high-density polyethylene membrane (Atemnkeng et al., 2006). The two 10% PVP-I formulations were found to have free iodine concentrations of 2.1 and 9.7 ppm, respectively, demonstrating a difference in bactericidal effect as found in free iodine concentration. They also measured free iodine concentrations in 50- to 100-fold diluted solutions from the two PVP-I formulations, reporting maximum values of 31 and 35 ppm, respectively. In our preliminary study using a permeation cell and a high-density polyethylene membrane, measurement of free iodine concentrations in PVP-I PB solutions and PVP-I formulations, diluted to optional concentrations revealed the maximum from the 100-fold dilution. Since this finding was consistent with reported results, a high-density polyethylene membrane was used as the permeation membrane for measurement of free iodine concentrations.

The free iodine concentrations in the 0.01, 0.1, 1 and 10% PVP-I PB at 25°C (ppm, mean±standard devia-

### TABLE 2. Virus inactivation effects (LRV) of 0.01-10% PVP-I PB on poliovirus type 1

| Test drug | Treatment time (min) | 0.25 | 1 | 5 | 15 | 30 | 60 |
|-----------|----------------------|------|---|---|----|---|----|
| PVP-I 10% | n.d.                 | n.d. | 0.5±0.5 | 0.6±0.5 | 1.1±0.1 | 2.4±0.1 |
| 1%        | n.d.                 | 0.6±0.1 | 0.7±0.3 | 2.5±0.1 | >4.2 | n.d. |
| 0.1%      | 0.6±0.2              | 0.6±0.2 | 2.6±0.3 | >4.2 | n.d. | n.d. |
| 0.01%     | n.d.                 | 0.6±0.2 | 1.7±0.4 | 3.6±0.4 | 4.2±0.1 | >4.2 |

Negative control (0.2M PB) n.d. n.d. n.d. n.d. n.d. 0.0

a: LRV = log10 (baseline viral infectivity ÷ viral infectivity obtained with each exposure time)
The LRVs were calculated from viral infectivity determined from 0.01-10% PVP-I PB solutions with various exposure times.
Each LRV value is expressed as the mean of two measurements. When both calculations are lower than the limit of detection, the LRV value is indicated as ">mean LRV."
For all other points, each value is expressed as the mean±standard deviation. As the negative control, only the infectivity titer following the longest exposure time with PB was measured.

b: Not determined.

### TABLE 3. Virus inactivation effects (LRV) of 0.01-10% PVP-I PB on adenovirus type 3

| Test drug | Treatment time (min) | 0.25 | 1 | 5 | 15 | 30 | 60 |
|-----------|----------------------|------|---|---|----|---|----|
| PVP-I 10% |                     | 0.1  | 0.6±0.1 | 1.5±0.5 | 2.7±1.0 | 3.7±0.8 | 4.3±0.3 |
| 1%        |                     | 0.7±0.3 | 2.4±0.7 | 4.3±0.3 | >4.3 | n.d. | n.d. |
| 0.1%      |                     | 2.6±0.6 | 4.3±0.5 | >4.3 | n.d. | n.d. | n.d. |
| 0.01%     |                     | 1.6±0.5 | 3.5±0.3 | >4.3 | >4.3 | n.d. | n.d. |

Negative control (0.2M PB) n.d. n.d. n.d. n.d. 0.2±0.2

a: LRV = log10 (baseline viral infectivity ÷ viral infectivity obtained with each exposure time)
The LRVs were calculated from viral infectivity determined from 0.01-10% PVP-I PB with various exposure times.
Each LRV value is expressed as the mean of three measurements. When all the three calculations are lower than the limit of detection, the LRV value is indicated as ">mean LRV."
For all other points, each value is expressed as the mean±standard deviation. As the negative control, only the infectivity titer following the longest exposure time with PB was measured.

b: Not determined.
The free iodine concentrations we obtained were lower than those reported by Atemnkeng et al. (2006). These differences are attributable to the differences in the choice of apparatus for membrane permeation and buffer concentration, as well as the measuring time, test solution pH and other conditions. However, the relationship between the free iodine concentration and available iodine concentration was determined generally agreed with their results. While the measurement of free iodine concentrations using membrane permeation cells is generally considered to be a useful method, given the extrapolated values outside of the regression line included in the results of this study, as well as the factors affecting the measured values, further study may be necessary on the measurement method in order to obtain and confirm highly accurate and sensitive measured values.

Generally, it has been reported that viruses having an envelope (outer lipid membrane) are highly susceptible to antiseptics, non-enveloped viruses are highly resistant to antiseptics and adenoviruses, which are lipophilic, possess relatively low resistance to antiseptics despite their identity as non-enveloped viruses (Prince and Prince, 2001). In the present study, influenza A virus was chosen from among enveloped viruses, and poliovirus and adenovirus from among non-enveloped viruses, on the basis of differences in viral structure and drug resistance and other factors. The 10% PVP-I solution prepared with purified water exhibited strong acidity (pH: 1.7), resulting in infectivity titer reductions due to the pH in two (influenza virus, poliovirus) of the three viruses used in the present study (data not shown). Taking this into consideration, a specific buffer was used to prepare PVP-I solutions; the buffer selection 0.2M PB and pH (pH: 7.0) was determined in accordance with a previously reported combination (Kawana et al., 1998). Prepared with the PB solution, a 10% PVP-I PB solution was found to have a pH of 6.7, demonstrating the absence of influence of the PB solution on any test virus.

The 0.01-10% PVP-I PB solutions were found to be effective in inactivating influenza virus at all concentrations examined by reducing the infectivity titer by ≥4 log reduction when allowed to act for 15 s, with no difference in virucidal efficacy observed among the different free iodine concentrations. On the other hand, the virucidal activity against poliovirus was found to differ among the different PVP-I concentrations; the virucidal efficacy of the PVP-I PB changed depending on the iodine concentration as follows: 0.1% ≥ 0.01% ≥ 1% > 10%. Similar results were obtained with adenovirus; the virucidal activity was found to differ among the different PVP-I concentrations, with the virucidal efficacy of the PVP-I PB changing depending on the iodine concentration as follows: 0.1% ≥ 0.01% ≥ 1% > 10%. With regard to susceptibility to PVP-I PB solutions, poliovirus was found to be the most highly resistant, followed by adenovirus and influenza virus in this order, agreeing with reported data on the susceptibility of existing agents (Prince and Prince, 2001).

Kawana and colleagues (1998) examined the virucidal efficacy of PVP-I PB solutions prepared to optional concentrations against various viruses, reporting that the virucidal efficacy against three enteroviruses, including poliovirus types 1 and 3, did not depend on the available iodine concentration in PVP-I, with the drug exhibiting weaker virucidal efficacy at higher concentrations than at lower concentrations (Kawana et al., 1998). Such a virucidal efficacy not depending on PVP-I concentration has been reported in rhinovirus, a non-envelope virus, and rubella virus, an envelope virus, as well. This fact appears to be consistent with our finding of the association between virucidal efficacy against non-envelope viruses and free iodine concentration, showing that the free iodine concentration was higher in the 0.1% PVP-I PB (available iodine: 0.01%) than in the 10% PVP-I PB (available iodine: 1%), and that the virucidal efficacy was found to depend on the free iodine concentration of each solution.

While PVP-I is known to exhibit antimicrobial activity proportional to the concentration of the free iodine released, our study using non-enveloped viruses verified that the virucidal efficacy was maximized at a concentration close to that of the 100-fold dilution (PVP-I concentration: 0.1%), which produced the highest free iodine concentration. In light of existing reports and the present findings, with no molecular species other than free iodine directly involved in virucidal efficacy, PVP-I is inferred to exist chiefly as a supply source of free iodine. Attention should be paid to the fact that PVP-I, unlike other antiseptics that lose antimicrobial activity with dilution, does not always lose antimicrobial activity even when having a decreased available iodine concentration due to dilution. Although 10% PVP-I formulations are commonly used in the clinical setting, taking into account concentration reductions due to the presence of organic substances such as blood and proteins, they should not be used unless the above-described features are fully understood.
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