Iodine-Mediated Inactivation of Lipid- and Nonlipid-Enveloped Viruses in Human Antithrombin III Concentrate


Human plasma-derived protein concentrates intended for clinical use must be treated for viral inactivation to ensure patient safety. This study explored the use of liquid iodine for inactivation of several lipid- and nonlipid-enveloped viruses in an antithrombin III (AT-III) concentrate. Iodine at levels of 0.01% to 0.02% caused between 43% and 94% loss of AT-III activity, as well as degradation of AT-III as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. However, addition of up to 0.1% human albumin protected the AT-III against both inactivation and fragmentation. At albumin levels sufficient to retain greater than 75% of AT-III activity, greater than 6 logs of sindbis, encephalomyocarditis, and vesicular stomatitis viruses, greater than 4 logs of pseudorabies, and greater than 3 logs of human immunodeficiency virus were inactivated. Except with sindbis virus, this represented complete inactivation of all the viruses spiked into the AT-III concentrate.

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MATERIALS AND METHODS

AT-III concentrate. The AT-III used was a pasteurized, freeze-dried concentrate purified from human plasma by heparin-Sepharose chromatography, according to the method of Wickerhauser and Williams.10 The concentrate was made for the American Red Cross by Baxter Healthcare Corp (Glendale, CA). After reconstitution, the product is in phosphate-buffered saline (PBS) at pH 7.3 and has an AT-III potency of 58 IU/mL, a protein concentration of 11.9 mg/mL, and a specific activity of 4.9 IU/mg. The product is essentially 100% AT-III by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Fig 1).

AT-III assay. AT-III concentration was determined as heparin cofactor activity using chromogenic substrate S-2238 (KabiPharmacia, Chromogenix Division, Franklin, OH) according to the method of Abildgaard et al,20 as modified by Wickerhauser and Williams.19 Aliquots from a pool of fresh-frozen human plasma, standardized against the First International Reference Preparation for AT-III,21 were used as a reference. Potency was expressed in international units (IU).

Electrophoresis, Coomassie staining, and Western blotting. Samples were separated under reducing and nonreducing conditions by SDS-PAGE on 8% to 16% gradient gels.22 The gels were stained with Coomassie Brilliant Blue R-250 (BioRad Laboratories, Rich-

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albumin; lane standards. Twenty micrograms pg albumin; lanes Amersham Corp. Arlington Heights, IL) in the nonreduced state for as described by Benade. I7 Western blots. SDS-PAGE-prestained molecular weight markers + mond, CA) or transferred to nitrocellulose filters (Hybond-ECL; then placed at 37°C in
mination. amino acid MEM with either

68). dilutions of mouse antihuman serum albumin polyclonal antibody cells (ATCC CCL
to the methods of Reed and Muench?'.

5% CO2. Results were read using an inverted microscope according to the methods of Reed and Muench.25
to the methods of Harada et al,23 as modified by Benade.27 The assay is based on the particular cytopathologic effects (appearance of syncytia) induced when these cells are infected by HIV. Briefly, six to eight serial 2× dilutions of the test sample were made across microtiter plates. MT2 cells were then dispersed, centrifuged, resuspended, counted, adjusted to a concentration of 2 × 10^5 to 3 × 10^5 cells per milliliter, and then seeded in 100-µL volumes into the wells of the microtiter plate. Plates were incubated at 37°C and monitored for activity on days 2 to 4, but the assay was typically scored on the fifth day by microscopic examination for wells containing evidence of cytopathic effects; specifically, the presence of readily identifiable syncytial cells. TCID_{50} calculations were performed by the method of Karber.23 Viral inactivation experiments. For VSV, SIN, PRV, and HIV inactivation studies, 1-mL stock suspensions of virus were diluted 1:50 in a human AT-III/PBS mixture (10.5 mg/mL AT-III) to yield titers of 6 to 8 log_{10}mL for VSV and SIN or 4 to 5 log_{10}mL for HIV and PRV. Subsequently, the AT-III plus virus mixture was diluted 1:5 with PBS (1 × PBS, Life Technologies Inc), resulting in a protein concentration of 2.1 mg/mL. For EMCV inactivation studies, 1 mL of a stock suspension of EMCV was diluted 1:100 in AT-III to obtain a final titer of approximately 10^8 TCID_{50} units of EMCV and a final AT-III concentration of 2.1 mg/mL. A stock solution of 2.5% albumin prepared immediately before use from albumin (human) US Pharmacopeia (Baxter Healthcare Corp) di-

Fig 1. SDS-PAGE under nonreduced (A) and reduced (B) conditions: lane 1, AT-III control; lane 2, 0.08% albumin; lane 3, AT-III + 0.08% albumin; lane 4, 0.08% albumin + 0.788 mmol/L ascorbic acid; lane 5, AT-III + 0.788 mmol/L ascorbic acid; lane 6, AT-III + 0.02% iodine + 0.788 mmol/L ascorbic acid; lane 7, 0.08% albumin + 0.02% iodine + 0.788 mmol/L ascorbic acid; lane 8, AT-III + 0.08% albumin + 0.02% iodine + 0.788 mmol/L ascorbic acid; lane 9, AT-III + 0.05% albumin + 0.02% iodine + 0.788 mmol/L ascorbic acid; and lane 10, molecular weight standards. Twenty micrograms of total protein was loaded in each lane. Lanes 1, 5, and 6 contain 20 µg AT-III; lanes 2, 4, and 7 contain 20 µg albumin; lanes 3 and 8 contain 14.6 µg AT-III plus 5.4 µg albumin; and lane 9 contains 16.6 µg AT-III plus 3.4 µg albumin.
Iodine exists in a number of different forms in aqueous solution: elemental iodine (I\(_2\)), hydroiodic acid (HI), iodine cation (H\(_2\)IO\(_3\)), triiodide ion (I\(_3^-\)), iodide ion (I\(^-\)), hypohalous acid (HO\(_X\)), and the iodate ion (IO\(_3^-\)), all of which are in pH-dependent equilibrium. However, only elemental iodine (I\(_2\)) is believed to play a vital role in viral inactivation. The solubility of iodine in water at pH 7.5 is approximately 330 mg/L at 25°C. To increase the I\(_2\) content of solutions, potassium iodide (KI) is added. Iodide (I\(^-\)) combines with I\(_2\) to yield triiodide, I\(_3^-\), which acts as a reservoir for the supply of I\(_2\) through the equilibrium reactions. Nevertheless, the concentration of the free I\(_2\), which is the active form, cannot exceed 330 mg/L at 25°C. To quench the iodine reaction, 1-ascorbic acid (vitamin C) is used in these experiments. The 1-ascorbic acid reduces I\(_2\) to I\(^-\), which is not reactive. Iodine is known to react with various functional groups on proteins and viruses. These include carbon-carbon double bonds on unsaturated fatty acids, amino groups on certain amino acids (histidine, arginine, lysine), oxidation of sulfhydryl groups (cysteine), reaction with phenol groups (tyrosine), and reaction with bases on nucleotides (cytosine, adenine, guanine). In reference to carbon-carbon double bonds, it is proposed that unsaturated fatty acids add to olefinic double bonds, which might result in changes in physical properties of the lipids and a decrease in fluidity of the cell membrane. With respect to amino groups and nucleotide bases, there is a tendency of iodine to bind to block potential hydrogen bonding sites, which could lead to changes in protein structure. In the case of cysteine becoming oxidized, it loses its ability to form disulfide bonds, which is known to play an important role in protein synthesis. In the case of phenol groups, it is known that iodine can bind in the ortho position and sterically hinder hydrogen bond formation. However, the reaction of iodine with proteins or peptides usually differs from that of free amino acids as a result of electronic or steric environments of the reactive centers. Thus, this leads to considerable variation in the susceptibility of individual residues to iodination.28

Both crosslinked starch-iodine (XL-starch-iodine) and cross-
linked polyvinylpyrrolidone-iodine (XLPVPI) have advantages as viral inactivating agents. For example, XL-starch-iodine and XLPVPI are easy and inexpensive to manufacture, have a wide range of particle sizes, and, consequently, can be tailored to a variety of chromatographic procedures. Both are easily removed, thus minimizing the risk of producing an adulterated product. Additionally, both tightly bind iodine, although XLPVP binds iodine less tightly than XL-starch. Conversely, not all situations require iodine to be tightly bound to a carrier. In fact, a liquid iodine solution can be more advantageous for inactivating viruses in protein concentrates. The use of liquid iodine eliminates the need to crosslink the carrier; thereby eliminating a very time-consuming step. Another advantage of liquid iodine is the higher initial concentration of free iodine for viral inactivation. Although it can be argued that higher iodine concentrations lead to higher losses of protein activity, we have shown that in the presence of albumin, greater than 75% of AT-III activity can be preserved.

Iodine sensitivity (eg, hyperthyroidism, hypothyroidism, thyroiditis, and goiter) is mentioned in the literature as far back as the late 1800s. In most cases, the sensitivity resulted from excess iodine in foods, dietary supplements, topical

**Table 2. Effect of Iodine Treatment on Viral Inactivation**

<table>
<thead>
<tr>
<th>Iodine (%)</th>
<th>Albumin (%)</th>
<th>Ascorbic Acid (mmol/L)</th>
<th>VSV</th>
<th>EMCV</th>
<th>SINV</th>
<th>PV</th>
<th>HIV</th>
</tr>
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<tr>
<td>0.0, controls</td>
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<td>0</td>
<td>ND</td>
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<td>0</td>
<td>ND</td>
</tr>
<tr>
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<td>0.394</td>
<td>0</td>
<td>ND</td>
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<td>ND</td>
<td>0</td>
<td>ND</td>
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<tr>
<td>0.0</td>
<td>0.394*</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>ND</td>
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<td>≥6.2</td>
<td>≥6.9</td>
<td>≥4.3</td>
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<td>0.394</td>
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<td>≥6.2</td>
<td>6.5</td>
<td>≤4.3</td>
<td>≥3.3</td>
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<td>0.394*</td>
<td>ND</td>
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<td>0</td>
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<tr>
<td>0.04</td>
<td>0.394</td>
<td>≥6.2</td>
<td>≥6.2</td>
<td>6.3</td>
<td>≤4.3</td>
<td>≥3.3</td>
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<tr>
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<td>0.394*</td>
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<td>≥6.2</td>
<td>≥6.9</td>
<td>≥4.3</td>
<td>ND</td>
</tr>
<tr>
<td>0.04</td>
<td>0.591</td>
<td>≥6.2</td>
<td>≥6.2</td>
<td>6.8</td>
<td>≤4.3</td>
<td>≥3.3</td>
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<tr>
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<td>0.591</td>
<td>≥6.2</td>
<td>≥6.2</td>
<td>≥6.9</td>
<td>≥4.3</td>
<td>ND</td>
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<tr>
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<td>ND</td>
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<td>≥6.2</td>
<td>≥6.9</td>
<td>≥4.3</td>
<td>≥3.3</td>
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<td>≥6.2</td>
<td>≥6.2</td>
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<td>6.8</td>
<td>≤4.3</td>
<td>≥3.3</td>
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Abbreviation: ND, not determined.

* Virus was added immediately after the 30-minute incubation and ascorbic acid neutralization.
noted, however, that the majority of people are not affected by excess iodine and that the quantity of iodine that is considered excess varies from person to person. Many studies done to determine population susceptibility to excess iodine may not be accurate because they deduced their results from a patient population already immunocompromised by disease. Additional information concerning iodide dose and response can be divided into four components: route of intake, bioavailability of iodine, duration of intake, and physiologic status of subject. Additionally, few controlled toxicity studies have been done, but are necessary to scientifically determine safe limits for iodine. On the other hand, it has been reported that iodide, the product from iodine conversion by albumin and ascorbic acid, is relatively harmless.

The data show that treatment with 0.01% to 0.02% iodine for 30 minutes at 24°C can cause complete inactivation of a number of model viruses in an AT-III concentrate. Iodine can also cause a significant loss in AT-III activity, as well as fragmentation of AT-III. This activity loss and fragmentation are mitigated significantly by the addition of albumin as a stabilizer and the use of ascorbic acid to quench the iodine reaction. Albumin and ascorbic acid also preserved a small amount of SIN V activity. The mechanism for protection by albumin/ascorbic acid is not currently known. However, it is likely that albumin acts as another target for iodine and thus reduces the effective concentration of iodine attacking AT-III and viruses.

These results are encouraging with respect to the use of iodine for viral inactivation in plasma products, but also raise several issues that must first be addressed. Activity loss is undesirable, but is also a characteristic of many other viral inactivation methods. For instance, AT-III is typically pasteurized for 10 hours at 60°C with 0.5 mol/L sodium citrate as a stabilizer, with a resulting activity loss of 8% to 30%. The resulting activity loss becomes part of the cost of the production process and is offset by the increased value of a product with reduced viral infectivity. Of more concern is whether the inactivated species remaining in the product have any detrimental effect to the patient. The heat-denatured AT-III remaining in pasteurized AT-III has never been observed to cause any untoward reaction in patients. The actual effect of iodine on AT-III and albumin will be the focus of future studies.

In summary, we report conditions in which greater than 6 logs of SIN V, VSV, and EMCV, greater than 4 logs of PRV, and greater than 3 logs of HIV can be inactivated while maintaining greater than 75% of the AT-III activity. Additional research is needed to determine the efficacy of iodine on actual human pathogenic viruses, such as hepatitis A and human parvovirus B19, as well as the effects of iodine on the product proteins.

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REFERENCES


Iodine-mediated inactivation of lipid- and nonlipid-enveloped viruses in human antithrombin III concentrate

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