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(54) **Titre** : UTILISATION D'AZOLES COMME AGENTS VIRUCIDES DANS DES SOLUTIONS DE PROTEINES BIOLOGIQUEMENT ACTIVES
(54) **Title:** USE OF AZOLES AS VIRUCIDAL AGENTS IN SOLUTIONS OF BIOLOGICALLY ACTIVE PROTEINS

(57) **Abrégé/Abstract:**
Soluble azoles in aqueous solutions can be used as virucidal agents for biologically active protein preparations.

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ABSTRACT OF THE DISCLOSURE

Soluble azoles in aqueous solutions can be used as virucidal agents for biologically active protein preparations.

BACKGROUND OF THE INVENTION

Field: This disclosure is concerned generally with methods of inactivating viruses in solutions of biologically active proteins and, specifically, with the use of azoles to inactivate viruses in an aqueous solution of biologically active therapeutic proteins.

Prior Art: The importance of eliminating viral infectivity in therapeutic products has long been recognized. This is especially true in the case of biologically active products derived from human blood or, more recently, from cell cultures used to make products of biotechnology (e.g., recombinant DNA products and monoclonal antibodies).

In considering virucidal agents for biologically active proteins, the primary goals are to assure complete virucidal action while not adversely affecting the biological activity of the protein. These goals require consideration of such variables as the protein itself, the nature of its activity and/or activity site, the virucidal agent, the importance and/or ease of its removal after use, and variables of the treatment itself, such as time, temperature and concentration.

Heat treatment alone can be used for virucidal treatment of some proteins (e.g., pasteurization at 60°C). However, it is difficult in many cases to avoid loss of biological activity or utility when heat alone is used.

To avoid some of the disadvantages or activity losses resulting from the use of heat alone, various chemical agents have been used or proposed as virucidal agents for biologically active proteins. See, for example, U.S. Patent No. 5,071,650, to G. Dove, M. Dobkin and M. Shearer, disclosing the use of alcohols under specific conditions.

The utility of nonexplosive organic solvent/detergent mixtures for the preparation of viral vaccines, and, more recently, for inactivation of endogenous viruses in preparations of biological products derived from human plasma, have been limited by conditions of use (e.g., pH). One virucidal compound used with biologically active proteins at neutral pH is tri-n-butyl phosphate (TNBP). See, for example, U.S. Patent No. 4,540,573 to A. Neurath and B. Horowitz, disclosing the use of TNBP to inactivate lipid-enveloped viruses. See also U.S. Patent No. 5,110,910 to G. Tsay disclosing the use of TNBP as a virucide under controlled pH, conductivity and protein concentration. Unfortunately, current virucides typically inactivate mainly lipid-enveloped viruses and have not been shown to be effective against a hardier class of viruses which lack lipid envelopes.

Various azole compounds have been used as fungicides (e.g., see U.S. Patent 5,006,513 to Hector, et al.), but we are unaware of any suggestion to use them as virucides in solutions of biologically active proteins, especially against non-lipid containing viruses. We now have found that azoles provide a unique ability to inactivate both enveloped and non-enveloped viruses in aqueous solutions while preserving the biological activity of proteins such as immunoglobulins. Details of our method are described below.

SUMMARY OF THE INVENTION

Our method of inactivating both enveloped and non-enveloped viruses in an aqueous solution of biologically active, therapeutic proteins comprises contacting the solution with an azole, under conditions sufficient to assure the substantial reduction of both classes of viruses (2 or more logs virus titer reduction for each class) without adversely affecting the biological activity of the proteins (i.e., greater than 50% recovery of activity). Virucidal activity may be enhanced by the presence of detergents. The primary functional group responsible for inactivation is the pentameric azole ring, which may be modulated by changes to the ring or by additional functional groups attached to the ring. A preferred azole is imidazole, preferably used with a detergent as described below.

In accordance with one aspect of the present invention there is provided a use of an azole for the inactivation of viruses in an aqueous solution of therapeutic, biologically active proteins under conditions sufficient to result in substantial viral inactivation without substantial adverse effects on the proteins.

In accordance with another aspect of the present invention, there is provided a method of inactivating both enveloped and non-enveloped viruses in an aqueous solution of therapeutic, biologically active proteins selected from the group consisting of coagulation factors and antibodies comprising the step of contacting the solution with an azole selected from the group consisting of imidazole, histidine, 2-imidazolidinone, and 1H-imidazole-4-ethanamine under conditions sufficient to result in the azole causing

a reduction of at least 2 logs virus titer and recovery of greater than 50% of biological activity of the proteins.

In accordance with still another aspect of the present invention, there is provided a use of an azole selected from the group consisting of imidazole, histidine, 2-imidazolidinone, and 1H-imidazole-4-ethanamine for the inactivation of enveloped and non-enveloped viruses in an aqueous solution of therapeutic, biologically active proteins selected from the group consisting of coagulation factors and antibodies under conditions sufficient to result in the azole causing a reduction of at least 2 logs virus titer and recovery of greater than 50% of biological activity of the proteins.

SPECIFIC EMBODIMENTS

Azoles have been found to demonstrate virucidal activity with both enveloped and non-enveloped viruses, as illustrated below. As used herein, the terms azole or azole analogs or azole derivatives refer to compounds having a pentameric azole ring and which have virucidal activity under conditions of temperature, pH and concentration which do not adversely affect the biological activity of a protein of interest such as a therapeutic protein.

MATERIALS AND METHODS

Chemical Agents

Azoles used were: imidazole, alpha-amino-1H-imidazole-4-propanoic acid (histidine), 2-imidazolidinone (ethylene urea), 1H-imidazole-4-ethanamine (I4EA). Azoles, detergents (e.g., polysorbate 80, Triton X-100), and buffer salts were reagent grade and obtained from Sigma Chemicals, St. Louis, Mo.

Virus

Enveloped:
Vesicular stomatitis virus (VSV), Indiana strain, obtained from the Finnish Red Cross, is a Rhabdovirus (RNA). Vaccinia virus, Lederle strain, ATCC VR-118, is a Poxvirus (DNA). Sindbis is a Togavirus (RNA). Herpes simplex virus type 1 (HSV-1) is a Herpesvirus (DNA).

Non-enveloped:
Encephalomyocarditis virus (EMC) is a murine picornavirus (RNA). Poliovirus type 2 is a human picornavirus (RNA). Reovirus type 3 virus is a reovirus (RNA). Derivation of viruses was reported previously (see, Lembach, et al., Current Studies in Hematology and Blood Transfusion. Basel, Karger, 1989; 56:97-108).

Virus Assay

All viruses except Sindbis were titrated under standard conditions on monolayers of VERO cells grown in 24 well plates using 4 wells per dilution. Titers are expressed in terms of tissue culture

infectious doses as a 50% end-point per mL ($TCID_{50}$/mL). Sindbis virus was titrated in similar fashion on monolayers of BHK-21 cells under standard conditions assessing cytopathic effects.

Proteins

Factor VIII (FVIII), a coagulation factor administered to hemophiliacs, was derived from recombinant baby hamster kidney cells grown in suspension culture. This recombinant FVIII product (rFVIII) is described in EP 160,457 in the name of D.J. Capon, et al.

Monoclonal antibodies (human) of class G (m-IgG, anti-tumor necrosis factor, cell line ATCC Accession No. HB9736, were derived from Epstein-Barr virus-transformed human B lymphocytes grown in suspension culture. IgG was purified to greater than 98% by ion exchange and size exclusion chromatography. These antibodies are described in EP 351,789 in the name of H. Kuo.

Fibrinogen was purified from human plasma by the Cohn-Oncley process. Human serum albumin was purified from human plasma by the Cohn-Oncley process (Fraction V). See, for example, Cohn, E.J., L.E. Strong, W.L. Hughes, D.J. Mulford, J.N. Ashworth, M. Melin and H.L. Taylor. "Preparation and Properties of Serum and Plasma Proteins. IV. A System for the Separation into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids." J. Am. Chem. Soc. 68, 459 (1946).

Protein recovery was evaluated by A280, radial immunodiffusion, and FPLC-Superose 6 (Pharmacia fast protein liquid chromatography by size exclusion). FVIII was assayed by a modified method of

Langdell (Langdell, R.D., Wagner, R.H., Brinkhouse, A. "Effect of anti-hemophiliac Factor on One-Stage clotting Test," J. Lab. Clin. Med. 41: 637-647, 1953) based on activated partial thromboplastin time (APTT).

Treatment Protocol

Protein solutions were seeded with a 1/100 dilution of stock virus to minimize the effects of virus-containing medium. A volume of azole solution was added to give a final desired concentration. Treated and control samples were incubated with intermittent gentle mixing under the specified conditions (time, temperature). Samples were removed at appropriate intervals and titrated immediately for residual infectious virus.

RESULTS

Studies were conducted to determine the types of azoles exhibiting virucidal activity. As seen in **Table 1**, a wide variety of azoles are effective in reducing VSV titer. The primary functional group resulting in virus inactivation is the pentameric azole ring, which may be modulated by changes to the ring or by additional functional groups attached to the ring.

Table 1

Inactivation Kinetics of VSV in FVIII containing a variety of azoles at 40°C.

Azole	Imidazole	Imidazole	Histidine	2-Imidazolidinone	I4EA
Concentration	0.02M	0.2M	0.2M	0.2M	0.2M
Time (hrs)	Virus Titer Remaining				
0	(7.5*)	(7.75)	(8.25)	(7.75)	(8.25)
1	--	6.25	7.0	>7.5	7.25
3	--	3.75	4.5	7.0	5.5
4	--	2.5	3.0	5.75	5.0
5	--	<1.5	2.5	5.75	4.25
6	5.75	<1.5	2.75	5.25	3.5

( ): untreated

*: $LOG_{10}TCID_{50}/ML$

--: not tested

Studies were conducted to determine the types of viruses susceptible to inactivation by azoles. **Table 2** lists conditions necessary to provide substantial virus reduction (2 or more logs reduction) for a variety of enveloped and non-enveloped viruses.

Table 2

Inactivation Parameters for a Variety of Enveloped and Non-Enveloped Viruses in FVIII.

	Enveloped Viruses				Non-Enveloped Viruses		
Virus	VSV	HSV-1	Sindbis	Vaccinia	Polio-2	EMC	REO-3
Treatment Time (hr)	0.25	0.25	<1	<1	7	4	7
Temperature (C)	15	15	15	40	40	40	40
Imidazole (M)	0.02	0.02	0.02	0.2	0.2	0.2	0.2
Triton™ X-100 (%)	0.05	0.05	0.05	0.1	0.1	0.1	0.1
Titer (initial)	8.0*	6.75	8.25	6.25	6.5	6.5	7.75
Titer (final)	<1.5	<1.5	<1.5	<1.5	4.5	1.5	4.25
Reduction	>6.5	>5.25	>6.75	>4.75	2.0	5.0	3.5

*: $LOG_{10}TCID_{50}/ML$

Detergents may enhance virucidal activity. **Table 3** lists several examples, demonstrating specific conditions of increased activity or lack thereof.

Table 3

Effect of Detergents and Temperature on Virucidal Activity of Imidazole.

Virus	Sindbis		EMC		
Temperature (C)	15		15		40
Imidazole Conc'n. (M)	0.02		0.2		0.2
Triton™ X-100 Conc'n. (%)	none	0.05	none	0.1	none
Time (hr)	**Virus Titer Remaining**				
0	8.25*	8.25*	6.5	6.5	6.5
0.5	--	2.0	--	6.0	5.7
1	8.0	<1.5	6.25	5.75	5.7
2			5.75	6.25	3.25
5			6.25	6.25	<1.5

*: $LOG_{10}TCID_{50}/ML$

--: not tested

A variety of proteins in aqueous solutions are not substantially affected by treatment with azoles. Recovery may vary, depending on the protein. For example, FVIII is a very large, labile protein. Conditions permitting substantial virus inactivation without loss of activity are uncommon. **Table 4** defines kinetics for FVIII.

Table 4

Protein Recovery in the Presence of Imidazole Under a Variety of Conditions.

Protein	FVIII	FVIII	FVIII	IgG	Fibrinogen
Imidazole Concentration (M)	0.02	0.2	0.2	0.5	0.2
Temperature (C)	40	30	40	40	40
Time		Protein Recovery (% of initial)			
0	100	100	100	100	100
1		91	85		
2			92		
3		83	86		
4		83	79		
5		84	78		
6		86	77		
7	>95	81	75		
24	>95			>95	>95

DISCUSSION

Speculation on how imidazole works is as follows:

Each virion consists of a protein coat which has many clusters of protein subunits. Subunits are stabilized in solution by metal ions and may dissociate when the metal concentration is reduced. The binding of protein subunits to metal ions is facilitated by the electron-donating side chains of residues such as histidine. A complex is formed between a metal ion and the epsilon nitrogen from the imidazole ring of a histidine residue on the protein. In virus-containing solution, free imidazole competes with the imidazole on the protein subunits. As metal ions bind to the free imidazole, the protein subunits become unstable and the virus coat is opened, thereby destroying the native structure of the virion. The phenomenon is also dependent on the concentration of protein (e.g., FVIII) in solution, further supporting that imidazole in solution competes with the imidazole on the protein.

Given the above disclosure it is thought variations will occur to those skilled in the art. Thus, the above examples should be construed as illustrative and the invention disclosed here should be limited only by the following claims.

CLAIMS:

1. A method of inactivating both enveloped and non-enveloped viruses in an aqueous solution of therapeutic, biologically active proteins selected from the group consisting of coagulation factors and antibodies comprising the step of contacting the solution with an azole selected from the group consisting of imidazole, histidine, 2-imidazolidinone, and 1H-imidazole-4-ethanamine under conditions sufficient to result in the azole causing a reduction of at least 2 logs virus titer and recovery of greater than 50% of biological activity of the proteins.

2. The method of claim 1, wherein the solution is contacted with imidazole or histidine at a concentration of between 0.2 and 0.5 M azole.

3. The method of claim 2, wherein the solution is contacted with imidazole or histidine for at least three hours.

4. The method of claim 3, wherein the solution is contacted with imidazole or histidine at a temperature of 30°-40°C.

5. The method of claim 1, wherein the solution is contacted with the azole in the presence of a detergent.

6. The method of claim 5, wherein the solution is contacted with imidazole or histidine.

7. The method of claim 6, wherein the solution is contacted with imidazole.

8. The method of claim 6, wherein the solution is contacted with imidazole or histidine at a temperature of 15°-40°C.

9. The method of any one of claims 5 to 8, wherein the detergent is Triton™ X-100 or polysorbate 80.

10. The method of any one of claims 5 to 8, wherein the detergent is Triton™ X-100.

11. Use of an azole selected from the group consisting of imidazole, histidine, 2-imidazolidinone, and 1H-imidazole-4-ethanamine for the inactivation of enveloped and non-enveloped viruses in an aqueous solution of therapeutic, biologically active proteins selected from the group consisting of coagulation factors and antibodies under conditions sufficient to result in the azole causing a reduction of at least 2 logs virus titer and recovery of greater than 50% of biological activity of the proteins.

12. The use of claim 11, wherein the solution is contacted with imidazole or histidine at a concentration of between 0.2 and 0.5 M azole.

13. The use of claim 12, wherein the solution is contacted with imidazole or histidine for at least three hours.

14. The use of claim 13, wherein the solution is contacted with imidazole or histidine at a temperature of 30°-40°C.

15. The use of claim 11, wherein the solution is contacted with the azole in the presence of a detergent.

16. The use of claim 15, wherein the detergent is Triton™ X-100 or polysorbate 80.