A Method Of Detecting Viral Contamination In Parenteral Solutions.

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A METHOD OF DETECTING VIRAL CONTAMINATION IN PARENTERAL SOLUTIONS

A Dissertation
Presented to
the Faculty of the School of Pharmacy
the University of the Pacific

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Joseph Alexander Woelfel
July 1978
This dissertation, written and submitted by

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Dated: July 25, 1978
A METHOD OF DETECTING VIRAL CONTAMINATION IN PARENTERAL SOLUTIONS

Abstract of Dissertation

The presence of contaminants in parenteral solutions is a constant nemesis against which pharmaceutical manufacturers, as well as medical, pharmacy, and nursing practitioners must vigilantly struggle to provide quality health care. At each level in the parenteral drug delivery system, contamination is possible before the patient actually receives the infusion. The implementation of better practices and procedures continues in the quest of contaminant-free parenterals. Nevertheless, the literature is replete with articles documenting contamination of parenteral medication.

Foreign body particulate matter has been found sequestered in the lungs of patients who have received intravenous therapy. The entrapment of foreign bodies can occur in other body organs besides the lungs. The hazardous effects of this particulate matter has been the subject of much concern. Other forms of parenteral contaminants have been reported in the literature. These include both bacterial and fungal contaminants.

Contaminant detection in parenteral solutions has been accomplished by several methods. These have included: visual inspection, nephelometric methods, methods of membrane filtration with subsequent microscopic examination, and methods employing various electronic adaptations.

No references have been published describing viral contamination of parenterals or methods for viral detection in parenteral solutions. Yet, viral contaminants infused directly into the blood of a patient may be of grave clinical significance. Thus, the objective of this project was to develop a method for detecting the presence of viruses in small and bulk parenteral solutions.

Both small and large volumes of Sodium Chloride Injection U.S.P. and 5 percent Dextrose Injection U.S.P. were inoculated with 100 I.U. or 1 I.U. of Tobacco Mosaic Virus (TMV) per ml of solution. The contents of these parenterals were concentrated to a retentate volume using molecular filtration. The retentate volume was examined for viral content using transmission electron microscopy with negative staining techniques.

Efficacy was determined by comparison of the results of the control groups of uncontaminated parenteral solutions with the contaminated test groups. Statistically significant differences were observed between the control groups, which were not subjected to the test method, and the test groups for both small and large volume parenteral solutions.

Efficiency, which denotes the viral contamination level at which viruses are detectable, was determined by comparing the control groups of uncontaminated parenteral solutions with contaminated test groups of the same solutions. Both groups were subjected to the test methodology. The control and the test groups showed statistically significant differences at the 100 and the 1 I.U. TMV contamination levels.

The results showed that the defined method of viral detection is efficacious and efficient at the tested TMV contamination levels. This method could probably be applied to the detection of other viral contaminants of parenteral solutions as well as to biological viral analysis methods.
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The author wishes to thank all those persons who have contributed in any way to this project or this dissertation.
To the author's parents and to Lillian R. Brughelli, this work is dedicated. No words can express the thankfulness and indebtedness of the author to these persons. Their contributions to the author's life and beliefs can never be measured.

Finally, the author dedicates his past, present, and future works and accomplishments to Him. For it is per Ipsum, et cum Ipso, et in Ipso, est tibi Deo Patri omnipotenti, in unitate Spiritus Sancti, omnis honor et gloria. Per omnia saecula saeculorum.
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INTRODUCTION

The presence of contaminants in parenteral solutions is a constant nemesis against which pharmaceutical manufacturers, as well as medical, pharmacy, and nursing practitioners must vigilantly struggle to provide quality health care. At each level in the parenteral drug delivery system, contamination is possible before the patient actually receives the infusion therapy. Through contemporary and future science and technology, the implementation of better practices and procedures continues in the quest of contaminant-free parenterals. Nonetheless, the literature is replete with articles documenting contamination of parenteral medication.

The objective of this research project is to supplement the quality control procedures for parenteral infusions with a method capable of detecting viral contaminants in small and large volume parenterals.
Contaminants in Parenterals

In 1949, Von Glahn and Hall (8) reported pulmonary emboli of cotton fibers which resulted from intravenous injection of fluids containing cotton fibers. At autopsy characteristic mononuclear infiltrates and multinucleated giant cells were found at the intimal sites of the foreign body lodgement. These incidental findings were corroborated by Konwaler's (9) report of pulmonary foreign body granuloma in autopsied cases having received intravenous therapy.

Subsequent articles appeared in the literature describing pulmonary arterial response to foreign bodies associated with intravenous therapy (13,14). Confirmatory experimental studies using animals were also conducted (27,28).

As shown by Liebow et al. (62) and Hales (61) arterio-venous shunts in the lung exist. When these are present, particulate contaminants of intravenous fluids may be delivered to the general circulation and then be sequestered in other body organs besides the lungs (26).

The impelling study of large volume parenteral contaminants originated in 1963 with the work of Garvan and Gunnar (1). These authors in their original article and subsequent articles (2,3) described: contamination levels of
intravenous fluids, the nature and origin of the contaminants, methods of particulate detection, the harmful effects of particulate contaminated intravenous fluids both in animal experiments and patients, and compendial recommendations for particulate detection, limits, and parenteral fluid manufacturing.

Resulting from the interest generated by the works of Garvan and Gunner, in July of 1966 the Federal Food and Drug Administration convened a Scientific Symposium on Large Volume Parenteral Solutions (64). The basic purposes of the symposium were to explore: the problems of parenteral solutions, the health significance of particulate matter, and the methods of minimizing intravenous fluid health hazards.

At this symposium, the findings of various authors (1-3, 10,13,14,27,28) were presented as evidence of hazardous effects associated with parenteral fluids. Jonas (12) described the potentially hazardous effects of introducing particulate matter into the vascular system. He stated that the effects of the injected particulate matter depend on three main factors:

1. the size, shape, and chemical characteristics of the particle,
2. the site of occlusion and degree of interruption of blood supply, and
3. the host response to the particle.

Included in the host response is the antigenic potential of the particulate matter. Lockhart (6) also addressed the
medical significance of particulate matter including inorganic particles, bacteria, and molds. Gross and Carter (5) described the pathogenic hazard of particulates in intravenous solutions causing pulmonary granulomatous inflammation. They stated that the medical significance of the particulate matter "rests essentially in a consideration of the state of patients receiving large amounts of parenteral fluids" and that recumbent states and concomitant therapy especially with large doses of corticosteroids are factors which influence the pathogenicity of contaminated infusions. Gross (7) in a subsequent article described the possibility of pulmonary arteriovenous shunts enabling particulate matter to obstruct systemic circulation causing diminished blood supply to vital organs.

Endicott et al. (10) in the symposium's proceedings related the significance, source, measurement, and elimination of particulate matter at the manufacturing level. Yakowitz (11) described the problems associated with the manufacture, storage, and use of large volume parenterals.

This symposium further stimulated interest in contaminants of large volume parenterals. Turco, Davis, and Sivelly (4) have quantitatively studied particulate matter in large volume parenterals with and without additives using a membrane filtration technique. They found that both additives and administration sets increased the number of particulates in intravenous solutions. These authors further
described the work of past investigators and related the use of a final filter set for removing particulate matter.

Turco and Davis (15) presented a thorough review of the literature on the clinical significance of particulate matter. Turco (33) has also described the hazards associated with parenteral therapy including microbial, pyrogenic, and other forms of particulate contamination in parenteral therapy.

The presence of particulate matter has been reported in commercial antibiotic injectables (23, 24, 29, 30). Rebagay et al. (23) have proposed that the residues in antibiotic preparations might react with tissue proteins and cause or contribute to phlebitis. Stewart (73) found traces of macromolecular proteins of peptide complexes in natural cephalosporins and penicillins which possessed allergenic potential. Thus, this type of particulate contaminant may elicit host immune response.

The clinical significance of particulate matter was also related in the Canadian Journal of Hospital Pharmacy (59). Duma has recently described the hazards of intravenous therapy in the New England Journal of Medicine (22) and has previously reported on particulate matter in the Annals of Internal Medicine (65). It is evident from the widespread literature reports that contaminated parenterals are now of unquestioned clinical concern.

Past works have described the possibility of microbial and fungal contamination of parenterals (6, 33). Numerous
reports now document the possibility of bacterial as well as mycelial contamination and some have proposed recommendations as to surveillance of contaminated large volume parenterals (19, 20, 21, 35, 36, 38, 39, 51-55, 58, 60). Duma et al. (60) cite cases of nosocomial septicemia from intravenous fluids and volume-control sets. Guynn et al. (35) found that 5 percent Dextrose Injection U.S.P. was the most hostile to bacterial growth, whereas Sodium Chloride Injection U.S.P., Lactated Ringer's Injection U.S.P., and 5 percent dextrose in Lactated Ringer's Injection were more conducive to bacterial growth. They found that gram-negative bacilli predominate over gram-positive cocci as the study of Duma et al. (60) had also indicated. In the work of Poretz et al. (20) most species of organisms isolated were commonly considered lacking virulence. Yet, these same organisms may be pathogenic in the debilitated, hospitalized patient. Furthermore, the elaboration of endotoxins by gram-negative bacilli presents another distinct threat to the patient from contaminated parenteral fluids. Wilkinson et al. (52) as well as Curry and Quie (63) have shown the preponderance and proliferation of Candida albicans in parenteral hyperalimentation solutions. Deeb and Natsios (58) stated that contamination of "in use" hyperalimentation fluids by Candida species occurs to an extent greater than 25 percent.

In 1971, the clinical significance of contaminated large volume parenterals was sadly realized. Over 50 patients
died as a result of infections which they received from contaminated screw-cap infusions (37,50). Resulting from this tragedy, the United States Pharmacopeial Convention, funded by the Federal Food and Drug Administration, organized the National Coordinating Committee on Large Volume Parenterals. Barker (45), project director and chairman of the Committee, has stated the Committee's purpose is to study the problems associated with large volume parenterals and coordinate efforts of the member organizations in dealing with these problems. Activity coordination, guidelines for problem identification, recommendations, and increased awareness of usage standards have resulted from the committee's endeavors (31,37,41,44,45,50).

Included in a recent report of the National Coordinating Committee on Large Volume Parenterals (44), it is recommended that an inline particulate matter retentive final filter be seriously considered as a requirement for the administration of large volume parenterals. This recommendation is based upon numerous studies and their conclusions as to the overall advantages of inline final filtration (16,17,32,34,38-40,42, 55,66,110,123). Some of the advantages arising from the use of inline filtration as reported in the above studies include:

1. the effective removal of particulate matter and resultant reduction of patient risk from these contaminants,

2. the decrease in microbial and pyrogenic contamination, and
3. the decrease in the incidence of phlebitis.

Some of the disadvantages of inline filters are:

1. reduced infusion flow rates especially with the 0.22 micrometer (μm) filters,
2. air entrapment in the filters stopping flow,
3. non-bacterial retention capabilities of filters used other than the 0.22 μm filters (38), and
4. the bothersome yet effective clogging of inline filters with particulate matter.

These inherent disadvantages can be overcome by the use of an air-venting, 0.22 μm inline filter with a large filtration surface as reported by Rapp et al. (42).

Contaminant Detection in Parenterals

The detection of particulate matter has been accomplished by several methods which are either nondestructive or destructive in nature (1-3,10,18,23,24,29,30,43,46,47,56,57,67).

Visual inspection under diffuse light has been used by manufacturers (56,57) and in hospital pharmacy admixture programs. This nondestructive method is subjective, qualitative not quantitative, and restricted to visualization of particles greater than 50 μm (56,57). Blanchard et al. (43) found this method inadequate for monitoring particulate matter in large volume parenterals.

Nephelometric methods use the Tyndall effect of visual examination by the light scattering potential of particulates in a beam of light. Particulate detection is dependent on the size of the contaminant and the angle of scatter.
Particles over one \( \mu m \) can be detected \((56, 57)\). This nondestruc
tive method provides qualitative comparison solution to solution. The method cannot distinguish types of particulate matter and is not quantitative. Garvan and Gunner \((1-3)\) and others \((67)\) have described its use.

Membrane filtration and subsequent microscopic examination is a destructive technique which has been used by numerous investigators \((1-3, 29, 30, 43, 57)\). This method provides a permanent record of particulate contamination and can provide both qualitative and quantitative comparisons. The method can be tedious and particles with refractive indices similar to the filter can go undetected. With the light microscope particles of 0.2 \( \mu m \) size can be resolved \((68)\). This method has been adopted by the United States Pharmacopeia for particulate matter determination in large volume injections for single-dose infusion \((72)\).

The Coulter Counter\(^a\) has been used for the indirect determination of particulate matter in parenteral infusions \((56, 69, 110)\). This method is classed as a destructive technique for particulate detection. It is a rapid, nonvisual method using the principle of changes in electrical resistance caused by particulate matter in an electrically conductive solution. Vessey and Kendall \((56)\) recorded particles as small as 2.0 \( \mu m \), yet it is possible to detect

\(^a\)Coulter Electronics, Hialeah, FL.
particles less than this size (10). The Coulter Counter can be directly used for electrolyte solutions only.

The Royco Liquid Counter\(^a\) is another instrument which has been successfully applied to particulate determination in injectable solutions (10,57,70). It is being used at the manufacturing level (70). As a batch sampler this instrument is categorized as providing a destructive method of indirect determination of particulates. The principle of operation is that of incident light scattering by contaminant particles in a flowing stream of the solution. Thus, it is similar to nephelometric methods, but the decrease in light resulting from a particle’s presence is actually measured by a light sensing cell and amplified to produce a characteristic pulse height (70). It provides a rapid means of particle detection for both electrolyte and non-electrolyte solutions (10,57). It is capable of detecting particles as small as two \(\mu\)m (70).

The Millipore TT MC Particle Measurement Computer System\(^b\) is an electronically automated adaptation of the membrane filtration and microscopic examination technique (57,71). The parenteral solution is filtered through a membrane filter. The filter with the collected particulate matter is then examined by a microscope connected to a

\(^a\)Royco Instruments, Menlo Park, CA
\(^b\)Millipore Corporation, Bedford, MA
television camera. The image of the particulate is processed by a computer module and displayed on a television monitor (71). As Lim et al. (57) have reported, this system "was more precise in detection and sizing of particles and more rapid in counting of particles than the microscopic manual examination method." The system is capable of measuring entire fields of particles or selective individual particles, counting entire fields, computing total area and average area of particulates, computing particle size distributions and other parameters (71). The resolution limit of the TT MC system is 0.2 μm, the limit of the optical microscope (68, 71).

Another instrument for the analysis of particulate matter in large volume parenterals is the Prototron. Draftz and Graf (46) and Blanchard et al. (43) have reported on this instrument, which uses a laser-light scattering principle. Sizing and counting of particulates are made on the parenteral contents in their container and thus the method is considered to be nondestructive. Both studies (43,46) reported the inherent advantages and disadvantages of the Prototron and stated that its use should be further investigated. As reported by Draftz and Graf (46), particles greater than one μm were recordable.

In a recent report Lantz et al. (47) have described

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*aThe Nucleopore Corporation, Pleasanton, CA*
the use of the HIAC Particle Counter\(^a\) for monitoring particulate matter in parenteral solutions. This instrument is a device using electronic stream scanning of particulates in the solution and operates on the principle of light blockage. It is capable of rapid and accurate counting of subvisual particles. The work of Lantz et al. described the advantages and disadvantages of this instrument.

Studies employing a membrane filtration technique followed by scanning electron microscopy have recently been presented in the literature demonstrating the value of this method for the examination of the topographical configuration of particulate matter (18, 23, 24, 109). Levinson et al. (18) used this method for the comparison of infusion particulates found in glass and plastic containers. Rebagay et al. (23, 24) used this technique to observe residues in antibiotic preparations. Winding and Holma (109) have recently reported the use of scanning electron microscopy and X-ray analysis of particles exceeding 0.2 \(\mu\)m. The X-ray analyzer coupled to the microscope could not chemically identify biological materials and other organic materials but could identify elements 9 through 93 in the periodic table.

**Viral Contamination of Parenterals**

Various types of contaminants have been found in parenteral fluids and many methods have been used in their

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\(^a\)High Accuracy Products, Clairmont, CA
detection. The articles published to date on large volume parenterals have reported contaminants with particle size above 0.2 \( \mu \text{m} \). The presence of particulates less than 0.2 \( \mu \text{m} \) has not yet been described for parenteral fluids. Thus, the nature of these contaminants or their clinical significance cannot be assessed.

A suspected contaminant of large volume parenterals in this submicron size range might be the virus. Viruses are reported to range from approximately 18 nanometers (nm), as exemplified by the Picornaviruses, to about 300 nm in size, as observed in the Poxviruses (98). The significance of the virus has and is still being recognized in both clinical medicine and epidemiology.

If viral contaminants exist in parenteral fluids, a grave and impending danger exists for the patient receiving this "therapy." A patient undergoing parenteral therapy undoubtedly receives it to facilitate correction of some pathological state. Such an ill person may be immunologically deficient at the time of therapy. As Gross and Carter (5) have stated, recumbent positions and concomitant therapy especially with large doses of corticosteroids are factors which influence the pathogenicity of contaminated infusions. With the present medical trends in transplants and corresponding use of immunosuppressants, viral contaminants infused directly into the blood stream of these patients may be clinically significant. In those patients who are immuno-
logically competent, a parenterally infused virus can cause disease (48). Furthermore, slow, inapparent and recurrent viruses can persist in their host without the clinical signs of disease, yet cause chronic degenerative maladies clinically expressed at future times (49). Thus, any human virus can be clinically significant when administered parenterally in an appropriate human host. Only speculation is possible about the health significance of parenterally infused non-human viruses.

The hypothesis that viruses may contaminate large volume parenterals receives support from the American Public Health Association's concern regarding the potential health hazards of waterborne viruses. This Association's International Conference on Viruses in Water was held in Mexico City in 1974 (99,111). Several recommendations for environmental health safety, research, and detection methods resulted from this conference (100,111). Articles have appeared in the literature regarding the presence and significance of viruses in water supplies (101-106). At present, neither parenteral manufacturers nor the Federal Food and Drug Administration's Parenteral Quality Control Laboratories routinely monitor parenteral contamination by viruses (112). Personnel at a Federal Food and Drug Administration's Quality Control Laboratory have expressed their belief that viruses could contaminate parenterals especially where aseptic filling, final sterilization by filtration, or parenteral admixture are performed (112).
Viral Contaminant Detection in Parenterals

The initial problem in the development of a method for the determination of virus contamination of parenterals is that of concentration or extraction of the virus from these solutions by an efficient means.

Chromatographic and membrane separation methods (92), molecular sieving methods (93), electrophoretic methods (94), ultracentrifugal methods (86-89), and filtration methods (90, 91, 107, 108) have been used successfully in virus concentration. Because of the quantity and the nature of the solutions studied, the filtration method is most amenable to efficient concentration.

The specific filtration procedure which affords virus concentration is molecular filtration. Molecular filtration has been described as a "technique for separating dissolved molecules on the basis of size by passing a solution through an infinitesimally fine filter. This molecular filter is a tough, thin, selectively permeable membrane which retains most macromolecules above a certain size, while allowing most smaller molecules, including solvent to pass into the filtrate" (108). Molecular filters are available with different retention abilities which are characterized by their nominal molecular weight limit. Molecules with molecular weights above this limit may be retained in a retentate volume, however the size and shape of the molecule also influence retention phenomenon. This nominal molecular weight limit serves as a guide to filter selection.
After filtration the retentate volume, usually less than three milliliters (ml), contains the concentrated virus. If it is necessary, the virus may be further concentrated by ultracentrifugation (107) or by further molecular filtration to even smaller retentate volumes (108).

Once virus concentration has been accomplished, electron microscopic examination of this concentrate using negative staining techniques allows visual detection of virus contaminants. Through initial electron microscopic examination, viruses can be classified into three general structural configurations: rod shaped, spherical, or tadpole shaped (98). As shown by Horne and Wildy (82,84), virion symmetry, especially capsid symmetry, is a useful criterion for virus classification. Negative staining methods for transmission electron microscopy have provided valuable information in the revelation and study of virus particles (74-85,96,97). The "drop method" as described by Haschemeyer and Myers (74) is the most amenable technique for the embedding of the specimen sample in the negative stain where viral concentrations may be low.
OBJECTIVES

The primary objective of this project will be to develop an accepted research method for determining the presence of viruses in small volume as well as bulk parenterals.

The following methods and procedures used to attain the objective of this endeavor will include:

1. defined volumes of the parenterals will be inoculated with specific quantities of Tobacco Mosaic Virus var. vulgaris U1, used as the model viral contaminant, to test the efficacy and efficiency of the detection method;

2. the contents of the selected parenterals will be concentrated to an appropriate retentate volume using molecular filtration through a filter of suitable nominal molecular weight limit, capable of retaining viruses;

3. this retentate volume will be examined for viral content using transmission electron microscopy with negative staining techniques;

4. defined volumes of Sodium Chloride Injection U.S.P. and 5 percent Dextrose Injection U.S.P. will be the parenteral infusions examined.
EXPERIMENTAL

Definitions

In this research endeavor, a method was developed to detect viral contaminants in parenteral solutions. This method was analyzed for its efficacy and efficiency. The term "efficacy," as used in this project designated the capability of viral detection by the method. The term "efficiency" denoted the viral contamination level at which viruses were detectable.

The Model Virus

The virus chosen for use as the inoculant for contaminating the parenteral solutions was Tobacco Mosaic Virus (TMV). This RNA virus is a plant pathogen possessing well defined rod shaped morphology and helical symmetry with respect to its nucleic acid core. The virus is approximately 300 nm in length and 17 nm in diameter (82). Most viruses studied to date have sizes within this range (98). Because TMV has characteristic geometry and virion dimensions representative of the major viral groups, this virus was used as the model virus for this study.

Tobacco Mosaic Virus is a chemically stable virus and shows an inactivation rate independent of pH in the range of
4.0 to 8.5 (113). A pure culture of the virus is stable in dilution in Sterile Water for Injection U.S.P. for about a year (114). These factors were advantageous for viral stability considerations in the parenteral solutions examined.

Fraenkel-Conrat (115) described a relationship between weight, viral particles, and plant lesions for TMV. One plant lesion is produced in Nicotiana tobaccum var. Xanthi for approximately every 0.1 nanogram (ng) or $10^6$ virus particles. Thus, one infectious unit (I.U.) corresponds to approximately $10^6$ virus particles for TMV (116).

In this experimentation, viral dilutions of pure stock cultures of Tobacco Mosaic Virus var. vulgaris UI strain, were prepared using molecularly filtered Sterile Water for Injection U.S.P. as a vehicle. The required volumes were micropipetted into sterile one ml glass stoppered volumetric flasks. The micropipette tips had been ethylene oxide

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[a] Obtained from T. Shalla, Ph.D., Dept. of Plant Pathology, University of California, Davis, CA.

[b] 142 mm Hi Flux U-F Cell equipped with a Pellicon PTGC membrane, 10,000 nominal molecular weight retention limit, available from Millipore Corporation, Bedford, MA.

[c] Abbott Laboratories, North Chicago, IL.


[e] Class A, Pyrex Brand, available from Dow Corning, Midland, MI.

sterilized\textsuperscript{a} prior to their use. All dilutions were prepared in a laminar air-flow area using a Class 100 high-efficiency particulate absolute filter.\textsuperscript{b}

Preparation and Monitoring of Molecularly Filtered Sterile Water for Injection

Throughout this investigation molecularly filtered sterile water for injection (MF SWFI) was used. This water was Sterile Water for Injection U.S.P. (SWFI) which had been subjected to molecular filtration to remove any particulate, bacterial, fungal, or viral contamination.

Liter (L) volumes of SWFI were filtered through a Pellicon molecular filter having a nominal molecular weight retention limit of 10,000. This filter was contained in a 142 mm Hi Flux U-F Cell. The solution was loaded into the cell by use of a standard intravenous administration set.\textsuperscript{c} A 15 to 20 psig.\textsuperscript{d} driving force was generated by dry grade nitrogen\textsuperscript{e} passed through a 25 mm, 0.2 \textmu m inline Fluoropore

\textsuperscript{a}AMSCO Cryotherm and Aerator, available from Amsco Industrial Co., Erie, PA.
\textsuperscript{b}GEN II, available from Plas-Labs, Lansing, MI.
\textsuperscript{c}Venoset-60, available from Abbott Laboratories, North Chicago, IL.
\textsuperscript{d}Pounds per square inch gauge.
\textsuperscript{e}Available from Union Carbide Corp., Linde Division, New York, NY.
filter. A Hi Flux U-F Cell agitator setting of two was used during the filtration. The filtrate was collected in stoppered, sterilized flasks and stored in a laminar flow hood.

All filtration equipment including accessories was cleaned, then ethylene oxide sterilized prior to use. Throughout the project any equipment not subjected to this type of sterilization was either dry heat sterilized at 160 to 170°C for at least three hours or autoclaved at 15 psig. and 121°C for the correct penetration time for the load. Chemical indicators were used to monitor the sterilization procedures.

The entire filtration procedure was performed in a laminar air-flow work area containing a Class 100 high-efficiency particulate absolute filter.

Whenever MFSWFI was prepared, contamination control

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a Millipore Corporation, Bedford, MA.
b Fleakers, available from Dow Corning, Midland, MI.
c Thelco Dry Heat Oven, available from Precision Scientific Group, GCA Corp., Chicago, IL.
d Model No. 999 - C, available from Wilmot Castle Co., Rochester, NY.
e Ethylene Oxide Indicator, available from Aseptic Indicator Co., North Hollywood, CA.
detection procedures were used. The stored filtrate was subjected to sterility testing for bacterial and fungal contamination. The following procedures were used:

At least 10 ml of MFSWFI were aseptically transferred to not less than 80 ml of sterile Fluid Thioglycollate Media\textsuperscript{a} (FTG) and to not less than 80 ml of sterile Sabouraud Dextrose Broth\textsuperscript{b} (SDB). At the same time blank controls, as well as contaminated controls were prepared. The blank controls detected contaminated media or septic procedures. The contaminated controls were used to test the media's ability to support bacterial or fungal growth. A contaminated bacterial control was prepared by inoculating not less than 50 ml of FTG with \textit{Bacillus subtilis}.\textsuperscript{c} A contaminated fungal control was prepared using \textit{Candida albicans}\textsuperscript{d} inoculated into not less than 10 ml of SDB. The bacterial contamination tests were incubated\textsuperscript{e} at 30 to 32°C, whereas the fungal contamination tests were incubated\textsuperscript{f} at 22 to 25°C. The tests were checked

\textsuperscript{a}Difco Laboratories, Detroit, MI.
\textsuperscript{b}Ibid.
\textsuperscript{c}Bakte Bennet Labs., Berkeley, CA.
\textsuperscript{d}Ibid.
\textsuperscript{e}Thelco Incubator, available from Precision Scientific Group, GCA Corp., Chicago, IL.
\textsuperscript{f}Ibid.
for growth daily for not less than 14 days.
The results of all tests performed showed no bacterial or fungal contamination of the MFSWFI or the blank controls. Growth occurred in all contamination controls.

To determine the presence of incidental viral contamination of the MFSWFI by TMV or any other virus, the following procedure was used:

The residual volume of SWFI remaining in the Hi Flux U-F Cell was removed by nitrogen pressure via the cannula attached to the cell. Sterile surgical tubing\(^a\) attached to the cannula enabled the collection of the sample in a sterile 20 by 150 mm Pyrex test tube.\(^b\) This residual volume was referred to as the retentate volume. Two to four drops of the filtrate, MFSWFI, were also collected in another sterile test tube. The inline Fluoropore filter used to filter the nitrogen was aseptically removed in the laminar air-flow hood. It was transferred to another sterile test tube containing 0.5 ml of MFSWFI. The retentate volume, filtrate, and inline filter collect volume were then prepared into negative stained specimens for transmission electron microscopy by the following procedure:

Two microliters (\(\mu\)l) of each solution were deposited

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\(^a\) Tygon tubing S 50-HL, available from Norton Plastics and Synthetics Division, Akron, OH.

\(^b\) Available from Dow Corning, Midland, MI.
on 400 mesh copper grids. In a laminar flow hood the grids had been substrated with Formvar and stabilized with a layer of vacuum evaporated carbon. The solutions were deposited on the grids by the use of sterile micropipettes. Two µl of a 0.5% w/v solution of uranyl acetate (UA) in MFSWFI were then deposited to the grid. This embedded the sample specimen in the UA negative stain. This procedure has been described by Haschemeyer and Myers (74) as the "drop method." Prior to the use of the UA solution, it had been filtered through a 25 mm PTGC Pellicon filter, possessing a nominal molecular weight retention limit of 10,000. The grid preparation was allowed to dry completely in the laminar air-flow environment. It was then examined in the transmission electron microscope at 50 or 60 KV and a magnification of at least 18,000.

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a Available from Ted Pella Co., Tustin, CA.
b0.2 µm Fluoropore filtered 0.25% w/v Formvar in Ethylene Dichloride.
cVarian Vacuum Evaporator, VE 10, available from Varian/Vacuum Division, Palo Alto, CA.
dAvailable from Drummond Scientific Co., Broomall, PA.
eMillipore Corporation, Bedford, MA.
fRCA TEM EMU3-BX, available from Radio Corp. of America, Camden, NJ.
gSiemens Elmiskop TEM IA, available from Siemens of America, Inc., New York, NY.
hKilovolts.
Throughout the project TMV was not detected in any of these control specimens for MFSWFI.

A procedural diagram of the preparation and monitoring of MFSWFI is presented in Figure 1.

**Parenteral Solutions**

In this study defined volumes of Sodium Chloride Injection U.S.P. and 5 percent Dextrose Injection U.S.P. were the parenteral solutions examined. Identical lots of the specified volumes of each solution were randomly chosen. These were then used throughout either the preliminary tests involving small volume parenterals or the tests with large volume infusions.

These two parenteral solutions were chosen because they or their varying combinations comprise the most commonly administered intravenous infusions.

**Preliminary Tests with Small Volumes of Parenteral Solutions**

**The Test Method**

A 10 ml aliquot volume was aseptically removed from a 50 ml volume of either Sodium Chloride Injection U.S.P. \(^a\) or 5 percent Dextrose Injection U.S.P. \(^a\) This volume was transferred to a sterile 16 mm by 125 mm test tube \(^b\) for each

\(^a\)McGaw Laboratories, Irvine, CA.

\(^b\)Pyrex brand, available from Dow Corning, Midland, MI.
Figure 1. Procedural Diagram of the Preparation and Monitoring of Molecularly Filtered Sterile Water for Injection.
analysis. This solution was inoculated with TMV by the use of a micropipette with a sterile tip. The virus was obtained from the serial dilutions of TMV already described.

After inoculation, the solution was gently agitated and then filtered using a Pellicon PT series molecular filter unit. The filter on this unit had a surface area of 11 cm² and a nominal molecular weight retention limit of 10,000. The solution was filtered by the use of a vacuum pump attached to a collection reservoir. The retentate volume remaining in the test tube after filtration was approximately 0.2 ml. The test tube and filter were then washed with a five ml portion of MFSWFI, again concentrated, then rewashed with another five ml volume of MFSWFI. This volume was again reconcentrated to 0.2 ml. After each filtration procedure, the filter was tested to ensure filter integrity as described by the manufacturer (120).

Two μl volumes of this retentate volume were deposited using a sterile micropipette on Formvar/carbon substrated 400 mesh copper grids. A two μl volume of 0.5% w/v uranyl acetate solution in MFSWFI was then placed on the specimen grid. For each test specimen four grid preparations were

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a Immersible Molecular Separator, available from Millipore Corporation, Bedford, MA.

b Square centimeters.

c Available from Millipore Corporation, Bedford, MA.
made. Two of the samples were allowed to dry completely on the grids and two were blotted with sterile filter paper after five minutes from the time of application of the stain. All procedures described above were conducted using aseptic techniques in a laminar air-flow environment.

To single blind the experiments, the specimens were examined for the presence or absence of TMV by an electron microscopy technician. Prior to this study, the technicians were instructed in the technique of negative staining and transmission electron microscopic observation of TMV.

Cleaning, Sanitizing, and Sterilization of the Molecular Filters

At the completion of each test using molecular filtration, the filter was cleaned with a one % w/v sodium hypochlorite solution for at least three hours. This solution was discarded after passing through the filter. At least 50 ml of MFSWFI were used to rinse the filter and the filter unit's inner matrix. The filter was then flushed with and placed in a two % w/v formaldehyde solution for not less than 14 hours. This formaldehyde solution was passed through the filter and discarded. At least 80 ml of MFSWFI were filtered through the membrane before its use in the next test. After every fifth test, a new molecular filter was selected and ethylene oxide sterilized prior to use.

Figure 2 diagramatically describes the test method as well as the cleaning, sanitizing, and sterilization procedures
Figure 2. Procedural Diagram of the Preliminary Test Method with Small Volumes of Parenteral Solutions.
used with small volumes of parenteral solutions.

Detection of TMV at Varying Viral Contamination Levels

Initially, it was necessary to identify approximate viral contamination levels which the test method could detect. The test method as described above was used for all of these determinations. The amount of the viral inoculum and the parenteral used in each test are shown in Table I.

The tests were first conducted using Sodium Chloride Injection U.S.P. The tests were then repeated using 5 percent Dextrose Injection U.S.P. at the lower contamination levels. These tests were concluded when a level of contamination in Sodium Chloride Injection U.S.P. was reached at which TMV could not be detected. The results are reported in Table I.

Based on the results of these tests, the 100 I.U. and 1 I.U. TMV contamination levels were chosen to evaluate the efficacy and efficiency of the test method. Suitable controls were established. Replications of each control and test treatment were performed to provide statistical validity.

Efficacy Determination at a Contamination Level of 100 I.U. of TMV per Milliliter of Solution

To determine the efficacy of the test method with small volumes of parenteral solutions at a contamination level of 100 I.U. of TMV per ml of solution, the following tests were performed:
### TABLE I

**PRELIMINARY TEST RESULTS**

**Detection of Tobacco Mosaic Virus in Small Volumes of Parenterals at Varying Viral Contamination Levels**

<table>
<thead>
<tr>
<th>Viral Contamination Level in Virus Particles (V.P.) and Infectious Units (I.U.) per Milliliter of Parenteral</th>
<th>Results Observed Using Sodium Chloride Injection U.S.P. (NS) and 5 percent Dextrose Injection U.S.P. (D5W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{10}$</td>
<td>10,000</td>
</tr>
<tr>
<td>$10^9$</td>
<td>1,000</td>
</tr>
<tr>
<td>$10^8$</td>
<td>100</td>
</tr>
<tr>
<td>$10^7$</td>
<td>10</td>
</tr>
<tr>
<td>$10^6$</td>
<td>1</td>
</tr>
<tr>
<td>$10^5$</td>
<td>less than 1</td>
</tr>
</tbody>
</table>

\(^a\) TMV observed.

\(^b\) TMV not observed.
Ten ml of Sodium Chloride Injection U.S.P. were aseptically removed from a 50 ml volume of this solution and transferred to a sterile 16 mm by 125 mm test tube. This solution was inoculated with 100 I.U. of TMV as performed in the initial tests. The solution was gently agitated to disperse the virus. Two µl of this mixture were then deposited on a Formvar/carbon substrated 400 mesh copper grid and negatively stained by the prior method. Electron microscopic examination followed. This procedure was repeated for a total of three replications with Sodium Chloride Injection U.S.P. and followed by three replications with 5 percent Dextrose Injection U.S.P. Thus, these contaminated solutions were examined for detectability of TMV without use of the test methodology. The results are found in Table II.

After these control tests, the same inoculation procedure with 100 I.U. of TMV was performed on the same volumes of the parenterals. These were then subjected to the filtration methodology described above in the initial tests. Three replications were conducted with Sodium Chloride Injection U.S.P. and then repeated with 5 percent Dextrose Injection U.S.P. Single blind electron microscopic examination was performed after the use of the test methodology. These results are also presented in Table II.
**TABLE II**

PRELIMINARY TEST RESULTS

Determination of the Efficacy of the Test Method with Small Volumes of Parenteral Solutions at a Contamination Level of 100 Infectious Units of Tobacco Mosaic Virus per Milliliter of Solution

<table>
<thead>
<tr>
<th></th>
<th>TMV Not Observed</th>
<th>TMV Observed</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examined Without Use of the Test Methodology</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Examined After Use of the Test Methodology</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

Calculated $P = 0.00108$
The data presented in Table II and all subsequent tables throughout this research were analyzed by the Fisher exact probability test (117).

**Efficacy Determination at a Contamination Level of 1 I.U. of TMV per Milliliter of Solution**

Once efficacy determination of the test method had been completed at a contamination level of 100 I.U. of TMV per ml of parenteral, the entire procedure described above was repeated. This procedure was repeated with a TMV contamination level of 1 I.U. per ml of solution. Using the same volumes and the same parenteral solutions, six replications were performed at this contamination level without the use of the test methodology. These were followed by six replications using the test procedure. Single blind examination was conducted on all specimens. The results are shown in Table III for contamination levels of 1 I.U. per ml of solution.

**Efficiency Determination at a Contamination Level of 100 I.U. of TMV per Milliliter of Solution**

The efficiency of the test method with small volumes of the parenterals at a contamination level of 100 I.U. of TMV per ml of solution was determined. Three replicates of 10 ml volumes of Sodium Chloride Injection U.S.P. and three replicates of 10 ml volumes of 5 percent Dextrose Injection U.S.P. were used in each test group.
TABLE III

PRELIMINARY TEST RESULTS

Determination of the Efficacy of the Test Method with Small Volumes of Parenteral Solutions at a Contamination Level of 1 Infectious Unit of Tobacco Mosaic Virus per Milliliter of Solution

<table>
<thead>
<tr>
<th></th>
<th>TMV Not Observed</th>
<th>TMV Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examined Without Use of the Test Methodology</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Examined After Use of the Test Methodology</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

Total 7 5 12

Calculated $P = 0.00866$
In the first group of tests, the control group, the parenterals were not contaminated with TMV. The second group of tests, the treatment group, was contaminated with 100 I.U. of TMV per ml for each test solution. Thus, one group was not contaminated and the other group was contaminated with the virus.

Each parenteral test specimen in a group was subjected to the filtration test method described in the efficacy determinations. The cleaning, sanitizing, sterilizing, sampling, staining, and examining procedures accompanying the filtration were used and remained unchanged. The results of electron microscopic examination of the specimens for the presence or absence of TMV are found in Table IV. A representative electron micrograph is provided in Figure 3.

**Efficiency Determination at a Contamination Level of 1 I.U. of TMV per Milliliter of Solution**

The efficiency of the test method was next determined using a contamination level of 1 I.U. of TMV per ml of the parenteral solution. The same parenterals, volumes, and replications were used here. The uncontaminated control and the contaminated treatment groups' observations are summarized in Table V. Figure 4 illustrates the typical findings upon electron microscopic examination.
TABLE IV

PRELIMINARY TEST RESULTS

Determination of the Efficiency of the Test Method with Small Volumes of Parenteral Solutions at a Contamination Level of 100 Infectious Units of Tobacco Mosaic Virus per Milliliter of Solution

<table>
<thead>
<tr>
<th></th>
<th>TMV Not Observed</th>
<th>TMV Observed</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncontaminated Parenteral Examined After Use of the Test Methodology</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Contaminated Parenteral Examined After Use of the Test Methodology</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

Calculated $P = 0.00108$
Figure 3. Electron Micrograph of Tobacco Mosaic Virus Detected at a Contamination Level of 100 Infectious Units per Milliliter of Small Volume Parenteral Solution (Magnification 98,000x)
TABLE V

PRELIMINARY TEST RESULTS

Determination of the Efficiency of the Test Method with Small Volumes of Parenteral Solutions at a Contamination Level of 1 Infectious Unit of Tobacco Mosaic Virus per Milliliter of Solution

<table>
<thead>
<tr>
<th></th>
<th>TMV Not Observed</th>
<th>TMV Observed</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncontaminated Parenteral Examined After Use of the Test Methodology</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Contaminated Parenteral Examined After Use of the Test Methodology</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>5</td>
<td>12</td>
</tr>
</tbody>
</table>

Calculated $P = 0.00866$
Figure 4. Electron Micrograph of Tobacco Mosaic Virus Detected at a Contamination Level of 1 Infectious Unit per Milliliter of Small Volume Parenteral Solution. (Magnification 98,000x)
Tests with Large Volumes of Parenteral Solutions

The Test Method

The test method for the detection of viral contamination of small volume parenterals used a molecular filtration membrane with an area of 11 cm². For bulk parenterals, a larger filtration membrane was needed to reduce filtration time. The Hi Flux U-F Cell, equipped with a 142 mm molecular filter with a nominal molecular weight retention limit of 10,000, was chosen to provide large volume filtration. The 142 mm membrane provided 158.37 cm² of filtration area. This cell, filter, and the accessories accompanying their use, have been described in the preparation of MFSWFI.

A one liter volume of either Sodium Chloride Injection U.S.P. or 5 percent Dextrose Injection U.S.P. was aseptically transferred to the cell by a standard intravenous administration set. The cell inlet portal was sealed and a 15 to 20 psig. driving force was applied by dry grade nitrogen passed through a 25 mm, 0.2 µm inline Fluoropore filter. Once the cell vent valve was closed, filtration began. During filtration a cell agitator setting of two was used.

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a Abbott Laboratories, North Chicago, IL.

b Venoset-60, available from Abbott Laboratories, North Chicago, IL.

c Available from Union Carbide Corp., Linde Division, New York, NY.

d Millipore Corporation, Bedford, MA.
The agitator prevented concentration polarization at the membrane surface, which would reduce the flow rate and alter the retention characteristics (108). The solution was concentrated to a volume less than three ml.

After this initial filtration procedure, the cell was aseptically filled with approximately 250 ml of MFSWFI. This volume was then filtered and concentrated to less than three ml. Again 250 ml of MFSWFI were aseptically added to the cell. This final rinse was filtered and concentrated to less than three ml. Just prior to the final concentration, about 0.2 ml of the filtrate was collected in a sterile test tube and retained for analysis.

The retentate volume in the cell was aseptically removed through the cell cannula by nitrogen pressure. The retentate volume was forced through the cell cannula, sterile surgical tubing, a and another cannula into a sterile 20 by 150 mm Pyrex test tube. b

Once this retentate volume was collected, the volume was further concentrated to approximately 0.2 ml. An 11 cm² Pellicon PT series molecular filter unit with a nominal molecular weight retention limit of 10,000 was used for this concentration procedure.

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aTygon tubing S 50-HL, available from Norton Plastics and Synthetics Division, Akron, OH.

bAvailable from Dow Corning, Midland, MI.
This retentate volume and the collected filtrate were subjected to the same negative staining procedure and single blind electron microscopic examination as described in the preliminary tests. The same volumes of the sample and stain as well as the same number of grid preparations per specimen were used.

All of the above procedures for the test method were conducted in a laminar air-flow hood.

**Cleaning, Sanitizing, and Sterilization of the Cell and the Molecular Filter**

After each test the 11 cm$^2$ molecular filter unit was cleaned with sodium hypochlorite solution and sanitized with formaldehyde solution. The procedure has been described in the preliminary tests. Following every fourth test, a new filter unit was selected. Prior to its use the new unit was ethylene oxide sterilized.

The Hi Flux U-F Cell and the 142 mm molecular filter were cleaned and sanitized in situ by the following procedure:

1. A 500 ml volume of one % w/v sodium hypochlorite was placed in the cell by use of a standard intravenous administration set. This cleaning solution was agitated in the cell and a small volume was allowed to pass through the filter. The remainder of the solution was retained in the cell for at least three hours. After this interval, the solution was filtered and discarded.
2. The cell was then rinsed with two, 250 ml volumes of MFSWFI. Each volume was agitated throughout the cell, then flushed through the filter.

3. Five-hundred ml of two % w/v formaldehyde solution were placed in the cell. This was followed by controlled agitation to ensure a uniform covering of the cell surfaces. A small volume of this solution was filtered through the membrane. Then the solution was retained in the cell for not less than 14 hours. At the end of this sanitizing procedure, the cell was flushed and the solution was discarded.

4. A 250 ml volume of MFSWFI was placed in the cell. The cell was thoroughly rinsed with the MFSWFI. The MFSWFI was then filtered through the membrane. This rinsing procedure was performed a total of four times to effectively remove traces of formaldehyde. The MFSWFI used for the fourth rinse was collected and analyzed for its formaldehyde content. The retentate volume was analyzed for residual TMV by the test method described previously. TMV could not be detected in this rinse retentate volume throughout the tests. The formaldehyde content in the rinse was analyzed by a procedure similar to the method described in the U.S.P. (118) and the method of Bricker and Johnson (119). The following procedure was used:

Five mg of chromotropic acid were dissolved in ten ml of a mixture of nine ml of Sulfuric Acid U.S.P. and four ml of distilled water. Five ml of this solution were
added to 0.2 ml of the formaldehyde rinse. The mixture was then heated for 10 minutes at 60°C on a water bath. A very faint violet color resulted. The amount of formaldehyde was determined spectrophotometrically\textsuperscript{a} at a wavelength to 570 nm by comparison with a reagent blank. A series of formaldehyde reference standards were prepared and analyzed. A Beer's Law plot was constructed. The average amount of formaldehyde detected in the fourth rinse was less than 3.5 micrograms (µg) per ml.

After the completion of every fourth test using the Hi Flux U-F Cell and its molecular filter, the cell was disassembled, cleaned with mild detergent, and thoroughly rinsed with copious volumes of distilled water. The cell and its components were then dried in a laminar air-flow hood. After drying, a new 142 mm molecular filter and a new inline Fluoropore filter were installed. The cell and all accessories were then ethylene oxide sterilized.

At this disassembly time, the Fluoropore inline filter was aseptically removed in the laminar air-flow hood. The filter was placed in a sterile test tube containing 0.5 ml of MFSWFI. The tube and its contents were agitated. Negatively stained specimens were then prepared for electron 

\textsuperscript{a}Perkin-Elmer Model 202 Spectrophotometer, available from Perkin-Elmer, Norwalk, CT.
microscopic examination. This procedure has been described in the preparation and monitoring techniques for MFSWFI. Throughout the experimentation TMV was not observed in these Fluoropore filter washings. This procedure was used to detect incidental contamination from the nitrogen source.

A procedural diagram of the test method including the cleaning, sanitizing, and sterilization methods is shown in Figure 5 for large volume parenterals.

Efficacy Determination at a Contamination Level of 100 I.U. of TMV per Milliliter of Solution

The efficacy determination tests performed with large volume parenterals were similar to those executed in the preliminary tests with small volume solutions. The control tests, which were examined without use of the test methodology, were conducted exactly as described in the preliminary tests using 100 I.U. of TMV in 10 ml aliquot volumes of the parenterals. However, the procedure was repeated for a total of four replications with Sodium Chloride Injection U.S.P. and followed by four replications with 5 percent Dextrose Injection U.S.P. The results of the control tests inoculated with virus but examined without use of the test methodology are described in Table VI.

Following these control tests, 1000 ml volumes of the parenterals were placed in the Hi Flux U-F Cell and inoculated with 100 I.U. of TMV per ml. Inoculation was performed by use of a micropipette with a sterile tip. The contaminated
Figure 5. Procedural Diagram of the Test Method with Large Volumes of Parenteral Solutions.
### TABLE VI

**TEST RESULTS**

Determination of the Efficacy of the Test Method with Large Volumes of Parenteral Solutions at a Contamination Level of 100 Infectious Units of *Tobacco Mosaic Virus* per Milliliter of Solution

<table>
<thead>
<tr>
<th></th>
<th>TMV Not Observed</th>
<th>TMV Observed</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examined Without Use of the Test Methodology</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Examined After Use of the Test Methodology</td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
</tbody>
</table>

Calculated $P = 0.000078$
solution was processed by the test method. Four replications with Sodium Chloride Injection U.S.P. and four replications with 5 percent Dextrose Injection U.S.P. were conducted. These results are summarized in Table VI. The 0.2 ml volume of filtrate collected at the end of each procedure did not demonstrate any TMV breakthrough from the molecular filter.

Efficacy Determination at a Contamination Level of 1 I.U. of TMV per Milliliter of Solution

After efficacy determination of the test method had been completed at a contamination level of 100 I.U. of TMV per ml of bulk parenteral, the entire procedure enumerated above was again repeated. For these tests a TMV contamination level of 1 I.U. per ml of solution was used. The results of the controls, examined without use of the test methodology, and the test treatment group, examined after use of the test method, are presented in Table VII. Again, the 0.2 ml volumes of filtrate collected at the end of each procedure showed no TMV breakthrough.

Efficiency Determination at a Contamination Level of 100 I.U. of TMV per Milliliter of Solution

The efficiency of the test methodology with large volumes of the parenteral solutions was determined. The contamination level of 100 I.U. of TMV per ml of bulk infusion was first examined. Liter volumes of Sodium Chloride Injection U.S.P. and 5 percent Dextrose Injection U.S.P. were used in the tests. Four replications of the test method for each of the two solutions were performed in a test group.
**TABLE VII**

TEST RESULTS

Determination of the Efficacy of the Test Method with Large Volumes of Parenteral Solutions at a Contamination Level of 1 Infectious Unit of Tobacco Mosaic Virus per Milliliter of Solution

<table>
<thead>
<tr>
<th></th>
<th>TMV Not Observed</th>
<th>TMV Observed</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examined Without Use of the Test Methodology</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Examined After Use of the Test Methodology</td>
<td>3</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>11</strong></td>
<td><strong>5</strong></td>
<td><strong>16</strong></td>
</tr>
</tbody>
</table>

Calculated $P = 0.017093$
The control test group of parenterals was not contaminated with TMV. The test treatment group was contaminated with 100 I.U. of TMV per ml in each bulk parenteral. The inoculation procedure was the same as that described in the efficacy tests. Each infusion, whether contaminated or uncontaminated, was subjected to the test method described previously. The results are reported in Table VIII and illustrated by a representative electron micrograph in Figure 6. No TMV breakthrough was found in the filtrate volumes collected.

**Efficiency Determination at a Contamination Level of 1 I.U. of TMV per Milliliter of Solution**

The efficiency determination method presented above was repeated at a contamination level of 1 I.U. of TMV per ml of the parenteral. The same parenterals, volumes, and replications were used for these tests. The uncontaminated and contaminated groups' results are recorded in Table IX. Figure 7 demonstrates the typical finding of electron microscopic examination. TMV breakthrough was not observed in the collected filtrate.
TABLE VIII

TEST RESULTS

Determination of the Efficiency of the Test Method with Large Volumes of Parenteral Solutions at a Contamination Level of 100 Infectious Units of Tobacco Mosaic Virus per Milliliter of Solution

<table>
<thead>
<tr>
<th></th>
<th>TMV Not Observed</th>
<th>TMV Observed</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uncontaminated Parenteral Examined After Use of the Test Methodology</strong></td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td><strong>Contaminated Parenteral Examined After Use of the Test Methodology</strong></td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
</tbody>
</table>

Calculated $P = 0.000078$
Figure 6. Electron Micrograph of Tobacco Mosaic Virus Detected at a Contamination Level of 100 Infectious Units per Milliliter of Large Volume Parenteral Solution (Magnification 98,000x)
TABLE IX

TEST RESULTS

Determination of the Efficiency of the Test Method with Large Volumes of Parenteral Solutions at a Contamination Level of 1 Infectious Unit of Tobacco Mosaic Virus per Milliliter of Solution

<table>
<thead>
<tr>
<th></th>
<th>TMV Not Observed</th>
<th>TMV Observed</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncontaminated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parenteral Examined After Use of the Test Methodology</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contaminated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parenteral Examined After Use of the Test Methodology</td>
<td>3</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>5</td>
<td>16</td>
</tr>
</tbody>
</table>

Calculated $P = 0.017093$
Figure 7. Electron Micrograph of Tobacco Mosaic Virus Detected at a Contamination Level of 1 Infectious Unit per Milliliter of Large Volume Parenteral Solution (Magnification 71,000x)
DISCUSSION

In this study both efficacy and efficiency were desired for the proposed test method of detecting viral contamination in parenteral solutions. The 100 I.U. and 1 I.U. TMV contamination levels identified in the preliminary tests enabled effective evaluation of the method. Using the preliminary test method, TMV was not detected at a viral contamination level less than 1 I.U. per ml of small volume parenteral solution. This was shown in the results presented in Table I.

For the preliminary tests with small volume parenteral solutions and the subsequent tests with large volume parenteral solutions, the controls used in the efficacy determinations of the test method were contaminated with either 100 I.U. or 1 I.U. of TMV per ml of parenteral solution. These controls were examined for the presence of TMV without the use of the test method. As reported in Tables II, III, VI, and VII, TMV could not be detected. When specimens using Sodium Chloride Injection U.S.P. were examined in the electron microscope, a dense granular precipitate was observed. This precipitate hindered viral detection. Upon examination of specimens using 5 percent Dextrose Injection U.S.P., similar results were obtained. The entire grid surface of the sample was electron dense. This opacity of the sample probably
resulted from caramelization of the dextrose in the electron beam. When the test method was used for the efficacy test treatment groups, viral detection was possible. The rinsing procedures and subsequent reconcentrations used in the test method reduced the concentration of the chemicals interfering with electron microscopic examination.

When using small volume parenteral solutions, the preliminary efficacy test results presented in Tables II and III demonstrated significant statistical difference between the control and the treatment groups. This was noted at both the 100 I.U. contamination level \( (P = 0.00108) \) and the 1 I.U. contamination level \( (P = 0.00866) \). In Tables VI and VII, the efficacy tests' results using large volume parenteral solutions showed significant difference between the control and the test treatment groups. This occurred at the contamination levels of 100 I.U. \( (P = 0.000078) \) and 1 I.U. \( (P = 0.017093) \). Thus, the efficacy of the test method for both small and large volume parenteral solutions was established.

The efficiency of the test method at contamination levels of 100 I.U. and 1 I.U. of TMV in small and large volume parenteral solutions was shown. Tables IV, V, VIII, and IX summarize this evidence. In the preliminary test and test control groups, incidental TMV contamination was not detected using the test methodology. Whereas in these same treatment groups, contaminated with 100 or 1 I.U. of TMV per ml of solution, TMV contamination was demonstrated by use of the
test methodology. For the preliminary tests with small volume parenteral solutions, statistically significant difference was observed between the control and treatment groups for the 100 I.U. level ($P = 0.00108$) and the 1 I.U. level ($P = 0.00866$). The tests using large volume parenteral solutions showed significant difference between the control and test groups. Efficiency of the test methodology for a TMV contamination level of 100 I.U. ($P = 0.000078$) and 1 I.U. ($P = 0.017093$) was demonstrated. The electron micrographs illustrated in Figures 3, 4, 6, and 7 describe the relative proportion of TMV occurrence as detected at 100 I.U. compared with the 1 I.U. level. Figures 3 and 6 show the characteristic clusters of many viruses usually observed at the 100 I.U. contamination level. At a contamination level of 1 I.U. of TMV per ml of solution, singly occurring TMV were usually detected. These results are typified in Figures 4 and 7.
SUMMARY

1. A survey of the literature on parenteral contaminants, methods of parenteral contaminant detection, viral contamination of parenterals, and methods of viral concentration was presented. In this literature review, no reference was found describing viral contamination of parenterals or a method for detecting viral contaminants in parenteral solutions.

2. A method was developed for determining the presence of viruses in small volume as well as bulk parenterals. The following methods and procedures were used to attain this objective:
   a. defined volumes of Sodium Chloride Injection U.S.P. and 5 percent Dextrose Injection U.S.P. were inoculated with 100 I.U. or 1 I.U. of Tobacco Mosaic Virus;
   b. the contents of the parenterals were concentrated to a retentate volume using molecular filtration;
   c. the retentate volume was examined for viral content using transmission electron microscopy with negative staining techniques;
   d. the test method was evaluated for its efficacy and efficiency through analysis of the data
by the Fisher exact probability test.

3. The results showed that the defined method of viral detection is efficacious and efficient at the tested TMV contamination levels for both small and large volume parenteral solutions.
CONCLUSIONS

A research method was developed for determining the presence of viruses in small volume as well as bulk parenteral solutions. The results of this experimentation showed that the defined method of viral detection is efficacious and efficient at the tested TMV contamination levels. This test method could probably be utilized for the detection of other viral contaminants of parenteral solutions. Furthermore, the retentate volume, collected after the concentration procedure in the test methodology, may be readily adapted to animal, egg, or cell-culture inoculation for viral infectivity analysis.

The test method may be classified as a destructive method of contaminant detection in parenterals since the contents of the parenteral must be removed from the container for examination. The method does require careful attention to the protocol and can be tedious. These disadvantages are inherent.

This test method is a direct method of viral contaminant detection and is a relatively rapid process after the initial material and equipment preparation. The information acquired may be the most significant advantage to be realized by use of the test methodology.
The test methodology might find use at various levels in the parenteral solution delivery system. Parenteral manufacturers could use this method to evaluate their products for the absence of virus at defined viral limits. Analysis of parenteral infusions for viral content might find use in hospitals for the evaluation of "in use" standards or monitoring of admixture techniques.

The expense of producing and administering a sterile, nonpyrogenic, and viral-free infusion could be justified for immunologically incompetent patients. Similarly, the expense incurred in developing a viral retentive inline final filter could be justified.

Future studies using this method or other more effective and efficient methods should be undertaken to pursue the assurance of viral-free parenteral solutions for improved health care.
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2. Ibid., 2, 1(1964).


100. Anon., ASM News (American Society for Microbiology), 41, 52(1975).


