

Use of Three Optical Systems to Obtain a
Microscopic Profile Of Asbestos And Other Non-Opaque Particulates

Larger Than 5 Micrometers In Parenteral Drugs

by

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*Cover page; page 4; page 5; page 6; page 7; page 8; page 11;
page 14 (Legends for figures); Figures numbered.*

Abstract

This report describes a comparative microscopic study of chrysotile fibers utilizing three sequential optical systems: plane polarized light (PL), phase-contrast (PC), and Nomarski differential interference-contrast (NDIC) for complementary image analyses. The introduction of NDIC for qualitative and quantitative determinations in this area of particulate analyses resolves the problem of diffraction haloes encountered in PC microscopy. High optical contrast, the instrumental characteristic of the NDIC, is particularly useful of the detection of chrysotile fibers at a magnification of 500X.

Introduction

In 1975, heightened awareness of the adverse clinical significance of particulates in animal and human circulatory systems (1) resulted in official regulations regarding parenterals. F.D.A. issued a directive restricting the use of fiber-shedding asbestos and/or glass fiber filters in the manufacture of parenteral drug dosage forms (2). The U.S.P. XIX sets standards in terms of number and size limits for particulate matter in large volume parenterals.

The purpose of this paper is to describe a microscopic method, applicable to parenterals, for monitoring non-opaque particulates in general, and fibers such as chrysotile asbestos in particular. Animal studies reported in the literature appear to indicate that the fibrous morphology and not the chemical composition of chrysotile fibers is of pathological significance (4,5). This paper describes a method of sample preparation and subsequent microscopic examination employing three optical contrast systems: Plane Polarized Light (PL), Phase-Contrast (PC), and Nomarski Differential Interference-Contrast (NDIC) for qualitative and quantitative characterization of non-opaque particulate matter.

Experimental

Atmospheric Conditions: All operations except microscopic examinations are conducted under Class 100 conditions (6).

Clearing reagents: A 1:1 solution of diethyl oxalate and dimethyl phthalate is filtered through a Millipore Mitex filter, pore size 5 micrometers. For each ml. of the solution 0.1 gram of white, ungridded mixed esters of cellulose membrane material is added (7). The mixture is stirred to dissolve the membrane material and set aside for a few days to eliminate air bubbles before use.

Microscopic Equipment: The standard [REDACTED] polarizing microscope (5)(4) was fitted with a revolving achromatic-aplanatic phase-contrast and interference-contrast condenser, type VZ, with top lens 1.4 N/A. The condenser is equipped with six stops: one for plane polarized light, two for phase-contrast, 16X-100X, and three positions for the planachromat objectives for the NDIC, viz., 16X, 40X, and 100X. The NDIC requires an interference-contrast slide which is inserted into the analyzer slot. The revolving nose-piece accommodates five objectives. The eyepiece contained a cross-hair micrometer.

Sample Preparation: Isolate particulates on a white, gridded, mixed esters of cellulose membrane filter (Millipore), pore size 0.45 micrometer as directed in the U.S.P. XIX, First Supplement (3).

As a final rinse, use 5 ml. of isobutyl alcohol which had been previously filtered through a 0.22 micrometer MF filter.

The filter must be thoroughly dried before clearing. Drying maybe accomplished by air (8 hrs) or by heating on a ^{Kofler} hot bench at about 90° for approximately one hour.

Microscopic Examination: In the sample preparation area place one drop of clearing reagent on a clean glass slide previously rinsed with trichlorotrifluoroethane. Position the filter on top of the drop so that only the entire lower surface is in contact with the reagent. Excess reagent should be avoided so that the top of the filter is never flooded. When the filter is completely transparent, cover the preparation with a clean trichlorotrifluoroethane-rinsed cover glass. The clearing reagent has a refractive index (N) of 1.46-1.47. The sample should be examined within a few days because crystallization of the clearing reagent occurs within a week. The entire filter is examined in white light at a magnification of approximately 500X, using three optical systems sequentially in the following order: PL, PC, NDIC. A darkened laboratory provides optimum conditions for the microscopist.

Comparative Microscopic Study of Chrysotile Fibers: For the purpose of illustration, Canadian chrysotile was mounted in immersion oil having a refractive index (N) of 1.464. The same microscopic field was photographed in PL, PC, and NDIC. The

focal plane in each system that exhibited the sharpest contrast was selected. *Figures 1, 2, 3 and if were photographed at* The magnification was 200X and the length of the principal bundle was 175 μ m, ~~for Figures 1, 2, 3, 4, 5.~~ *In these figures,* The rounded masses on either side of the midpoint of the specimen are foreign crystals.

Figure 1. Viewed in PL with uncrossed polars, chrysotile fibers appear as a skein of transparent, ~~cylindrical~~ very flexible parallel fibers with random waves, curls and twists. Although the edge effects (diffraction, reflection, and refraction) provide dark contrast, the fibers characteristically provide a blurred image due to the fact that the fibril bundle does not lie in one focal plane. Glare and slight haloes degrade the image. Chrysotile is dichroic and absorbs most strongly in the γ index position, i.e., the length of the fiber parallel to the vibrational direction of the polarizer. In this position thick fibers may show green to blue-green colors and appear yellow-green to colorless in the α position. Thin fibers exhibit no color, however, a slight change in intensity is usually discernible with the fiber transparent in the α position and somewhat opaque in the γ position.

Figure 2. The chrysotile bundle is viewed in PL with polars crossed and placed in the position of maximum brightness. The birefringence of chrysotile is very low (0.004-0.016) and very thin fibers, *less than 0.5* ~~one~~ micrometer in diameter, are invisible.

White to first order yellow is the maximum interference color range for thick fibers. The extinction is parallel and the sign of elongation is positive. The first order red compensator can be used for the detection of ^{very} thin fibers that were invisible with crossed polars. Fibers aligned parallel to the slow ray of the compensator show second order blue color and exhibit good contrast against the magenta background.

Figure 3 and 5. The chrysotile bundle in Figure 3 and anthophyllite fiber in Figure ⁵ are viewed in PC. Diffraction haloes around the specimen and ghost images of particles above and below the focal plane are in part instrumental characteristics of PC. Note ghost images in the lower portion of the southwest quadrant of Figure 3. The large optical path discontinuities between the refractive index of chrysotile and the surrounding medium plus the wedge-edge effect of the combined haloes of the individual fiber bundles make halation a severe problem. In the microscopic field, only thin, well separated fibers are viewed to advantage.

Fibers that did not present useful images when viewed with the first order red plate are seen in good contrast in PC. Thin fibers in positive PC are seen as dark threads which usually exhibit some terminal end-flair of the fibrils. Thick chrysotile particles appear as opaque masses such as the dark mass positioned at approximately three o'clock in Figure 3. Careful focal studies of this particle (in PC) barely suggested its tight

fibrous mass. This particle, when well focused in the NDIC system shows the topography of a fibrous mass.

The observations made in the discussion of the chrysotile fibers in Figure 3 apply to the PC appearance of anthophyllite in Figure ⁵~~5~~.

⁴~~5~~ Figure ⁴~~5~~ and 6. The chrysotile bundle and anthophyllite fragment are viewed in NDIC. The sharp optical discontinuities that produced halation in the PC image appear in dark and light contrast, at the specimen-medium boundaries. The chrysotile fibers are perceived by their shadow cast slopes and appear three dimensional. The specimen's variation in optical path (Refractive Index x Thickness) across the width of the fiber bundle is visually translated as elevations of various heights. In NDIC microscopy, interfaces between media of different refractive index appear in relief, high relief for areas of higher index and depressions for the lower index. The topography of the chrysotile bundle seen in the NDIC image reflects the gradient of phase difference in the transverse direction which is parallel to the direction of shear of the Nomarski prisms. This contrast is differential interference-contrast. The diagonal, north-east to south-west in the microscopic field is the direction of shear. Specimens of chrysotile and amphiboles also effect a "super-modulation" of the light to give amplitude-contrast (8) when oriented perpendicularly to the direction of shear. Thus the fibers are clearly delineated and appear of brighter intensity

than the background. A large fragment of anthophyllite is seen in Figure 6.

Microscopic differentiations between such amphiboles as amosite, crocidolite, and anthophyllite could not be made.

Discussion

The cleared membrane filter presents a background which exhibits ^{uniform} ~~some granulation, of even pore size.~~ Two other clearing procedures suggested by the Millipore Corporation have not been tested. They are the use of immersion oil (refractive index $(N) = 1.515$) (9) and the transformation of the filter into a transparent hard plastic (10). The principal problem encountered on dissolution of the filter was due to residual moisture in the filter. Faulty clearing is noticeable as a visible turbidity and for such filters microscopic resolution is destroyed. An additional problem was the friability of the inked grid markings. Excess pressure in applying the cover glass to the filter can cause the opaque grid particles to migrate. Under these circumstances a relatively large grid fragment can cause light blockage and thus degrade the image of coincident or adjacent particles in the field.

The sequential use of the three optical systems, PL, PC, and NDIC provides maximum contrast and resolution for the morphological identification and optical crystallographic characterization of non-opaque particulates in the fixed refractive index (N) medium of 1.46-1.47. The arrangement of these optical

systems on the standard polarizing microscope represents a practical approach to the selection of a contrast technique which furnishes the most qualitative information concerning a particulate. For the examination of a microscopic field which may contain particles of various sizes and compositions, each system provides some information and one system will exhibit optimum contrast. Compare Figures 7 and 8. In Figure 7 one focal plane in PC provides a good contrast image of the diatomaceous earth fragment. The coincident chrysotile fiber in this field cannot be sharply focused in any one plane. On the other hand, Figure 8 shows the field of Figure 7 rotated for optimum NDIC contrast. The image of the chrysotile fiber is sharper and halation is negligible.

PC is very useful for phase objects having an optical path difference (Refractive Index x Thickness) of $\lambda/10 - \lambda/2$. Larger differences introduced halation. The majority of particulates encountered in parenteral drug analyses are of a higher refractive index than the medium and are seen in dark contrast on a lighter background in the Zeiss positive PC. The NDIC is applicable in a broader path difference range ($\lambda/10 - 1\lambda$) and has a very shallow depth of field which provides sharp images free of spurious contrast above and below the focal plane. This instrumental characteristic is very useful for optical sectioning of thick specimens and in instances of overlapping particulates.

Instrumental factors are significant and deserve some comment. There are no instrumental drawbacks to the PL system. Low and high power objectives can be used when the appropriate condenser top lens element is employed. Lenses with 0.63 - 1.4 N/A are available. The optical components of the PC system are rotation-symmetric and specimen orientation in the field is not significant. This is not the case in the NDIC system. There is a fixed orientation of the instrument; viz., the specimen may be oriented in a position 90° with respect to the direction of shear and then the stage is rotated so that the specimen is observed in a position parallel to the direction of shear. There is an azimuth effect through this angle and some features of a specimen may appear more pronounced in one position than another. Although the planachromat objectives used the NDIC are not centerable on the revolving multiple nosepiece, a single centerable nosepiece is available from the [REDACTED] for perfect centration of the objective. The less than perfect centration of the specimen in the field can be corrected during rotation by manual manipulation using the mechanical stage. In practice, for routine work, this adjustment is often not critical.

(b)(4)

Sources of particulate contamination of parenteral drugs can be broadly categorized. Algae, molds, insect fragments and animal hairs are introduced under unsanitary conditions.

Tyvek fibers (DuPont) from clean room garments and starch grains from powdered gloves are possible laboratory contaminants. Diatomaceous earth, glass wool, asbestos and cellulose fibers may be residual particles from production line filters. Many non-opaque particulates can be readily identified in the PL system if encountered in a typical form. If however found in a degraded state, identification becomes more difficult. Two examples of particulates found in an atypical state are hydrolyzed starch grains and very fine shredded cellulosic fibers. A hydrolyzed corn starch grain is isotropic when viewed between crossed polars in PL. This grain when viewed in NDIC clearly shows the depressed centric stellate helium characteristic of corn starch. A shredded cellulosic fiber 1 micrometer in diameter often twists and curls, and may strongly resemble chrysotile in the PL and PC systems. However, when observed in the NDIC it does not effect a "super-modulation" of the light and does not present the fibrillar, cylindrical profile which is characteristic of chrysotile.

The method described was initially employed for the detection of chrysotile and amphibole asbestos but was found to be very useful for the examination of other non-opaque particulates of low birefringence. The inherent characteristics of the NDIC system was found to enhance those aspects of the PL and PC microscopic images which were deficient in contrast.

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Legends For Figures

Figure 1. Canadian chrysotile^{photographed} at 200X magnification; principal bundle is 175 um in length; plane polarized light.

Figure 2. Canadian chrysotile^{photographed} at 200X magnification; same field as in Figure 1; plane polarized light with crossed polars.

Figure 3. Canadian chrysotile^{photographed} at 200X magnification; same field as Figure 1; phase contrast.

Figure 4. Anthophyllite^{photographed} at 500X magnification; phase contrast. *long axis of the largest fragment is 57µ*

Figure 5. Canadian chrysotile^{photographed} at 200X magnification; same field as in Figure 1, long axis of the fiber bundle oriented perpendicular to the direction of shear; Zeiss/Nomarski differential interference-contrast.

Figure 6. Anthophyllite^{photographed} at 500X magnification; long axis of the largestst fragment (57 um) is oriented perpendicular to the direction of shear; same field as in Figure 4; Zeiss/Nomarski differential interference-contrast.

Figure 7. Coincident chrysotile fiber and diatomaceous earth fragment^{photographed} at 500X magnification; phase contrast.

Figure 8. Coincident chrysotile fiber and diatomaceous earth fragment^{photographed} at 500X magnification rotated for maximum contrast of the fiber; same field as in Figure 7; Zeiss/Nomarski differential interference-contrast.