A comparative study on the performance of asbestos-free depth-filters for removal of pyrogens from infusion fluids

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Three asbestos-free depth-filters were compared with traditionally used asbestos-containing depth-filters for chemical integrity, physical integrity and pyrogen retention. With chemical and physical integrity only minor differences between the various filter types were seen. One asbestos-free depth-filter, based on charcoal, showed poor pyrogen-retention; this might be due to a high flow rate used. Asbestos-free depth-filters based on kieselguhr or on a mixture of kaolin and alumina proved to be good alternatives; these filters have proved to be suitable for the removal of pyrogens from electrolyte- or carbohydrate-containing infusion fluids.

Asbestos, which has long been the primary component of depth-filters used in the pharmaceutical industry for pyrogen-filtration, is merely a collective noun for a variety of minerals. These can be divided into two groups: chrysotile (serpentine) and amphibole. 90% of all asbestos, including that which is in use in depth-filters consists of chrysotile (Ampian 1976). Chrysotile consists of $3\ MgO.2\ SiO_2.2\ H_2O$. In neutral solutions asbestos has a positive zeta-potential. The unique filtration capacity of the chrysotile fibre is caused by its hollow-fibre structure which leads to a large surface area per weight (Yada 1967).

Capture of particles in depth-filters occurs in two ways: (i) by mechanical obstruction or (ii) by electrokinetic attraction between particles and filter-medium. This attraction is optimal when the filter-medium has a positive zeta-potential since most particles have a negative charge (Fiore & Babineau 1979).

The occupational hazards of the use of asbestos are well-known and have recently been reviewed by Brodeur (1980). Smith et al (1965) have shown that parenteral administration of asbestos can lead to neoplasms in animals. Asbestos fibres in parenteral drugs were found by Nicholson et al (1972). The release of these fibres by filters into the filtrate is described as media migration. This can vary broadly during a filtration process as a consequence of particle loading and pressure changes (Dwyer 1975).

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Of all asbestos varieties, chrysotile, the main component of asbestos-depth-filters, is considered the most toxic (Allison 1971).

The Food and Drug Administration (Federal Register 1975) has now prohibited the use of fibre-releasing filters for the production of large volume parenterals. This has led to increasing research for new filter materials suitable as depth-filter media.

Depth-filtration however is not the only way to remove pyrogens from solutions. Ultrafiltration is also effective (Zimmerman et al 1976; Sweadner et al 1977), but until now, ultrafiltration as a means of pyrogen removal was only suitable for small-scale preparation, e.g. up to 400 litres/5 m^2 h^{-1} (Zimmermann et al 1976).

We have investigated the suitability of some asbestos-free depth-filters, especially for large-scale preparations, and compared the results with those of the traditionally used asbestos-filters. The following parameters have been studied: (i) chemical integrity; migration of soluble agents into the filtrate; (ii) physical integrity; migration of particles into the filtrate; (iii) pyrogen retention from pyrogen-contaminated solutions.

The terminology concerning pyrogens is often confusing. Pyrogens are fever-inducing agents; those usually encountered in infusion fluids are of bacterial origin and have been chemically characterized as lipopolysaccharides (See also Galanos et al 1977; Sullivan et al 1976). We used *Escherichia coli* endotoxin to investigate the pyrogen-retention capacity of the filters.

MATERIALS AND METHODS

The filters involved were: (i) a filter containing asbestos (Seitz EKS); (ii) a filter containing kieselguhr (Seitz EKS Supra); (iii) a filter containing kaolin and alumina (Filtrox AF-S-steril); (iv) a filter containing charcoal (Seitz AKS). The commercially available infusion fluids (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) used as test solutions were: water for injection, NaCl 0·9%, dextrose 10%, Ringer's solution (U.S.P.).

Chemical and physical integrity

Water for injection (Ph.Eur.) was filtered through 0·2 m^2 filtration area (five 20 × 20 cm plates in a Seitz Pilot filtration apparatus) with a constant rate of 80 litre h^{-1} using pressurized air with a pressure of approximately 2·5 bar. Samples were taken: (i) from the fluid to be filtered before the beginning and at the end of the filtration; (ii) from the filtrate after filtration of 3, 10, 25, 50 and 90 litres.

Samples were collected in bottles (type II glass), rinsed thoroughly with water for injection and then sterilized for 20 min at 121 °C. They were tested for the following parameters:

(i) pH. The pH was measured using a Philips PW 9408 Digital pH-meter.

(ii) Conductivity was measured to establish the total release of soluble conducting substances, such as inorganic salts, using a Philips PW 9501 conductivity meter; results were transformed into and expressed as resistance.

(iii) U.v. absorption in the 350–200 nm range caused by the release of organic substances.

(iv) Particulate matter. Media migration was measured using a Royco Particle Counter Model 366. In 50 ml of the sample the numbers of particles with diameter of 2—5 μm, 5–10 μm, 10–20 μm, 20–50 μm and > 50 μm were counted.

(v) Oxidizable matter. Oxidizable matter present in the filtrate was determined as another indication for the release of organic substances. We used Kübels method: to 100·0 ml of the filtrate 5 ml 5 M H_2SO_4 and 10·00 ml 0·002 M $KMnO_4$ were added. The solution was then covered with a watch-glass, boiled for 10 min, and then 10·00 ml 0·005 M oxalic acid added. While still warm the solution was titrated with 0·002 M $KMnO_4$ until the red colour was stable (a ml). At the same time a blank was done by titrating 10·00 ml 0·005 M oxalic acid with 0·002 M $KMnO_4$ (b ml). Then 10·00 + a − b ml 0·002 M $KMnO_4$ has been reduced by the oxidizable substances present in the filtrate.

Filtrations for studying chemical parameters were done in triplicate for each filter type. Filtrations for measuring media migration were done in quintuplicate for each filter type for statistical reasons. Results are expressed as mean ± s.e.m.

Pyrogen retention

Pyrogen retention was examined by using a small-scale filtration apparatus with a filtration area of 42 cm^2. After the filter had been rinsed with 0·3 litre endotoxin-free test solution, 500 ml test solution containing 100 ng ml^{-1} E. coli endotoxin (Byk-Mallinckrodt) was filtered at a rate of 25 ml min^{-1} using pressurized air with a pressure of 0·5–0·8 bar.

Samples were taken after filtration of 50, 100, 250, 500 ml and were collected in pyrogen-free test-tubes (Byk-Mallinckrodt). The amount of endotoxins in the samples was determined quantitatively by a method, which is a modification of the Limulus Amoebocyte Lysate test, first described by Iwanaga et al (1978), in which a chromogenic substrate is used to indicate the lysate activation (Visser et al, in preparation).

In principle: endotoxins activate a pro-enzyme present in the lysate and this activated enzyme initiates the formation of a gel. The activated enzyme also catalyses the cleavage of p-nitroaniline (pNA) from the substrate S 2423 (CH_3-CO-Ileu-Glu-Gly-Arg-pNa; a generous gift of Kabi AB, Mölndal, Sweden). pNA formation can be measured spectrophotometrically at 405 nm.

A 10-test vial of Limulus Amoebocyte Lysate (Mallinckrodt) was reconstituted with a mixture of 1·2 ml Tris-buffer 0·4 M (pH = 7·0) and 1·6 ml water for injection. 70 μl of this reagent was preincubated with 50 μl of the sample for 45 min at 37 °C. 100 μl of this preincubated mixture was added to 500 μl of a mixture of 1·4 ml chromogenic substrate solution (3 m M) and 8·6 ml buffer solution (130 m M Tris, 3·28 mM $MgCl_2$, pH = 9) and the rate of the pNA-formation monitored spectrophotometrically at 405 nm at 37 °C. The increase in absorption min^{-1}, corrected for the blank, was calculated and compared with endotoxin-standards (0–50 pg ml^{-1}). Where necessary proper dilutions of the sample were made.

The sensitivity of this method depends on the quality of the lysate, the endotoxin content of the blanks, and a possible inhibition of the reaction by medium-constituents (NaCl 0·9% and Ringer's solution slightly inhibit). The detection limit in these experiments ranged from 5–10 pg ml^{-1}. For the sake of simplicity we consider 10 pg ml^{-1} as the detection limit.

Experiments were in triplicate for each solution and for each filter type.

Statistics

Results of the tests for chemical integrity were submitted to analysis of variance to establish significant differences between the various filter types. Results of the tests for physical integrity were tested for significance by the variance ratio test.

Differences were considered to be significant at the $P < 0.05$ level.

RESULTS

Chemical integrity

Samples taken from the fluid to be filtered before the beginning and at the end of the filtration did not show any differences with respect to the parameters being studied. Therefore no mention is made of the results of the latter samples.

(i) *pH*. Kieselguhr and kaolin filters released significantly larger amounts of alkalinic substances (Fig. 1). No significant difference was found between the kieselguhr and the kaolin filter nor between the asbestos and the charcoal filter.
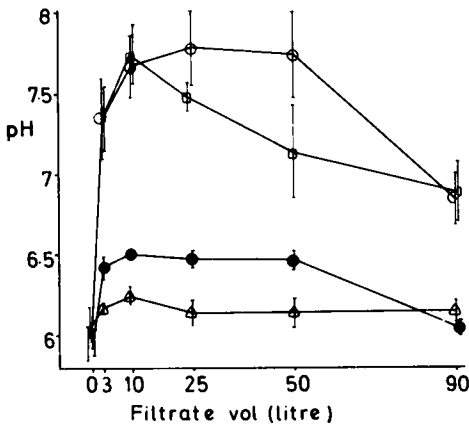
FIG. 1. pH of the filtrate after filtration of 90 litres water for injection through 0·2 m² filtration area. O= kieselguhr filter, □ = kaolin filter, ● = asbestos filter, △ = charcoal filter.

(ii) *Conductivity*. All three asbestos-free depth-filters released significantly larger amounts of conducting materials than did the asbestos filter (Fig. 2). No significant differences were found between the asbestos-free depth-filters.

(iii) *U.v.-absorption*. Of all samples only the 3 litre sample of the kaolin filter and the 3 litre sample of the kieselguhr filter contained u.v.-absorbing compounds with an absorbance ranging from 0·04–0·09.
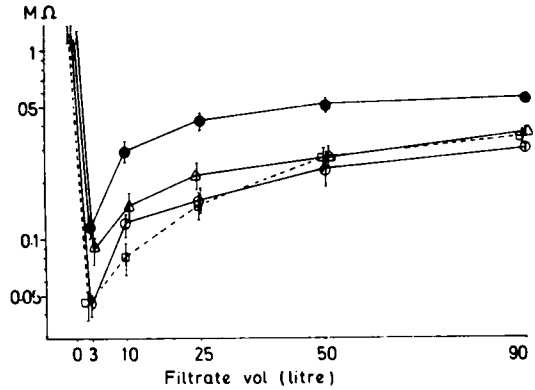
FIG. 2. Electrical resistance of the filtrate in Mohms after filtration of 90 litres water for injection through 0·2 m² filtration area. O = kieselguhr filter, □ = kaolin filter, ● = asbestos filter, △ = charcoal filter.

None of the other samples showed any detectable u.v.-absorption.

(iv) *Oxidizable matter*. The highest release of oxidizable substances was in the filtrate of the asbestos filters (Fig. 3), only the charcoal filter did not release oxidizable material. Differences between the charcoal filter and the others were significant. After filtration of 10 litres the filtrates from all filters were within the limit of oxidizable matter in water for injection (2·00 ml 0·002 M $KMnO_4$ per 100 ml, Ph.Eur.).
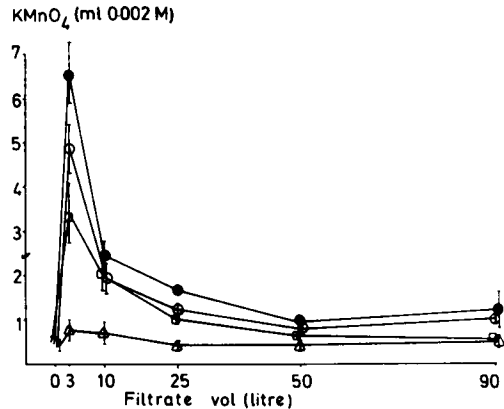
FIG. 3. The amount of oxidizable matter in the filtrate after filtration of 90 litres water for injection through 0·2 m² filtration area. O = kieselguhr filter, □ = kaolin filter, ● = asbestos filter, △ = charcoal filter.

Physical integrity

Results of the experiments for testing the delivery of particles into the filtrate are shown in Table 1. Again no differences were observed between samples from

Table 1. Number of particles (mean ± s.e.m.) in 50 ml filtrate after filtration of 90 litres water for injection through 0·2 m² filtration area. (n = 5).

Filter type and filtrate volume (litres)	Number of particles in 50 ml				
	2–5 μm	5–10 μm	10–20 μm	20–50 μm	> 50 μm
Asbestos					
0	1382 ± 229	522 ± 127	94 ± 25	24 ± 10	5 ± 3
3	1448 ± 335	600 ± 167	143 ± 47	65 ± 32*	12 ± 8*
10	1628 ± 229	771 ± 136	162 ± 37	49 ± 20	7 ± 3
25	1700 ± 249	777 ± 147	191 ± 41	69 ± 30	6 ± 2
50	1719 ± 247	733 ± 98	144 ± 28	40 ± 7	5 ± 1
90	267 ± 62*	141 ± 47	98 ± 43	52 ± 30	14 ± 8*
Kieselguhr					
0	1110 ± 187	489 ± 108	62 ± 22	25 ± 13	3 ± 2
3	2467 ± 618	807 ± 241	158 ± 57	30 ± 9	6 ± 3
10	1717 ± 476*	597 ± 125	145 ± 35	39 ± 14	5 ± 4
25	1321 ± 603*	634 ± 309	144 ± 68*	29 ± 16	3 ± 1
50	861 ± 326	387 ± 157	88 ± 30	23 ± 14	1 ± 1
90	610 ± 335	297 ± 195	95 ± 80	39 ± 2	3 ± 3
Charcoal					
0	1469 ± 343	740 ± 247	135 ± 56	48 ± 15	11 ± 4
3	7255 ± 1610*	2787 ± 837*	642 ± 263*	143 ± 44	17 ± 6
10	1916 ± 374	808 ± 195	176 ± 52	44 ± 12	6 ± 2
25	655 ± 115	282 ± 48*	59 ± 12*	16 ± 7*	2 ± 1*
50	813 ± 160	383 ± 109	113 ± 47	35 ± 16	3 ± 1*
90	612 ± 173	229 ± 60*	49 ± 14*	13 ± 4*	2 ± 1*
Kaolin					
0	859 ± 219	440 ± 184	109 ± 66	26 ± 15	2 ± 1
3	56538 ± 19174*	27470 ± 12813*	3370 ± 2016*	110 ± 43*	3 ± 1
10	5949 ± 1143*	2175 ± 487*	297 ± 60	51 ± 18	6 ± 2
25	1839 ± 338	863 ± 191	195 ± 54	48 ± 16	7 ± 2
50	1342 ± 247	660 ± 135	186 ± 41	54 ± 20	6 ± 2
90	671 ± 164	336 ± 80	99 ± 30	50 ± 41	5 ± 5

* $P < 0.05$.

the fluid to be filtered before the beginning and at the end of the filtration.

Significantly larger amounts of particles were seen only in the 3, 10, and 25 litre samples of the various filters. Other samples contained significantly lower amounts of particles, especially the 25 and 90 litre samples of the charcoal filter. Possibly the charcoal filter possesses a high filtration-capacity for these particle-sizes.

Pyrogen retention
The results of the pyrogen retention efficiency experiments are shown in Table 2. This efficiency is indicated throughout by lower concentrations in the filtrate.

In the first set of experiments, when water was used as test solution, the charcoal filter showed poor pyrogen retention. This could be expected with the flow-rate used (see also Discussion). Therefore we decided not to experiment further with this filter.

Both the asbestos and the kieselguhr filters showed an increasing efficiency as filtration continued when Ringer's solution was used. This also occurred with the asbestos filter and the NaCl 0·9% solution. We have no explanation for this.

However, we were not able to detect any release of endotoxin-like substances by these filters into the filtrate; a decrease of this release during filtration could result in an apparent increase in filtration efficiency.

We repeated the experiments with water for injection for the asbestos, the kieselguhr and the kaolin filter in duplicate with a tenfold higher endotoxin load (1000 ng ml^{-1}). All samples were below the detection level.

DISCUSSION
Chemical and physical integrity
The results of the tests for chemical integrity demonstrate only minor differences, although statistically significant, between the asbestos-free alternatives and the traditionally used asbestos filters. The kieselguhr and the kaolin filters show an increased release of alkalinic substances compared with the others. Oxidizable matter is eluted very quickly from all filters. The extraction of soluble conducting agents appears with all filters during the filtration. This extraction was significantly higher for the asbestos-free alternatives. There can be no doubt that the charcoal filter contains the lowest amount of

Table 2. Pyrogen retention of the various filters during the filtration of test solutions containing 10^5 pg ml^{-1} E. coli endotoxin (n = 3).

Test solution	Filtrate volume (ml)	Endotoxin content (mean ± s.e.m.) of filtrate (pg ml^{-1})			
		Asbestos	Kieselguhr	Kaolin	Charcoal
Water for injection	50	< 10	< 10	< 10	$30 \pm 2 \times 10^3$
	100	< 10	< 10	< 10	$29 \pm 5 \times 10^3$
	250	< 10	< 10	< 10	$29 \pm 2 \times 10^3$
	500	< 10	< 10	< 10	$41 \pm 4 \times 10^3$
NaCl 0·9%	50	130 ± 8	< 10	13 ± 2	—
	100	140 ± 60	< 10	< 10	—
	250	23 ± 10	< 10	< 10	—
	500	< 10	< 10	11 ± 1	—
Dextrose 10%	50	< 10	< 10	< 10	—
	100	< 10	< 10	< 10	—
	250	< 10	< 10	< 10	—
	500	< 10	< 10	< 10	—
Ringer's solution	50	330 ± 70	3500 ± 1500	12 ± 6	—
	100	140 ± 45	5300 ± 400	28 ± 15	—
	250	< 10	2400 ± 760	11 ± 4	—
	500	< 10	140 ± 50	11 ± 3	—

extractables; the liberation of oxidizable matter and alkalinic substances especially, is remarkably low.

With regard to the physical integrity there were no significant differences between filter types except for small particles in the beginning of the filtration. However, the sensitivity of the method was reduced by high background counts and a large variation between counts from individual samples. It is well-known that glass-bottles do release glass-spicules when autoclaved or stored for any length of time (Groves & de Malka 1976). This might explain the high background particulate matter contamination. However, when compared with the official standards for particulate matter in injections, these counts were low. These standards are given in Table 3.

The results of the tests for physical integrity do not lend support to the statement of Groves & de Malka (1976) that filtration is a source of particulate matter. With respect to the membrane filtration which is usually done to capture fibres released by the asbestos filters, it can be said that it has not yet been proven that membrane filtration can bring about a 100% removal of particulate matter released by depth-filters (Manalan 1974).

In the interpretation of these data it has to be borne in mind that rinsing of the filters is a normal part of a depth-filtration procedure. It is striking

Table 3. Official standards for particulate matter in injections.

	Particle size	No of particles ml^{-1}
U.S.P. 1975	> 10 μm	< 50
	> 25 μm	<5
British Pharmacopoeia 1973	> 2 μm	< 1000
	> 5 μm	< 100

that, for the charcoal filter, a larger rinsing volume (200 litre m^{-2}) is advised compared with that advised for the kieselguhr filter (50–100 litre m^{-2}). No data with respect to this are given by the manufacturer of the kaolin filter. From our results, a rinsing volume of 200 litres m^{-2} seems reasonable for all filter types.

Pyrogen retention

From the results it appears that the efficiency of the filters depends, in part, on the composition of the solution. This is not surprising since it is well-known that endotoxins can appear in different states of aggregation depending on the medium.

The highest aggregation state is a bilayer-sheet or vesicle with a diameter of 0·1 μm (Sweadner et al 1977). These structures are broken down to smaller aggregates (micelles) or even sub-units when EDTA or bile-salts are added to the solution (Ribi et al 1966; DePamphilis 1971). It is also known that differences in biological activity exist between these aggregation states, the sub-units do not show any biological activity (Ribi et al 1966). Possible conformations of these sub-units have been described (Hannecart-Pokorny et al 1973). Divalent cations (Ca^{2+}, Mg^{2+}) especially contribute to the aggregation but monovalent cations also have a profound influence on the physicochemical properties of lipopolysaccharides (Galanos et al 1977).

In these experiments different results were seen for the various solutions, Ringer's solution which contains Ca^{2+} ions had a negative effect on the filtration efficiency of two filter types. We have no explanation for these differences, possibly a high aggregation state results in less adsorption onto the filter or different compositions have a profound

influence on the electrokinetic forces between the filter medium and endotoxins.

The endotoxin load we used in these experiments is fairly high. The PD50 (pyrogenic dose) for *E. coli* endotoxin in the rabbit, the sensitivity of which equals that of man, amounts to 11 ng kg^{-1} (Cooper et al 1971). This means that in the U.S.P. Pyrogen test for infusion fluids, where 10 ml kg^{-1} is injected, those products which contain about 1 ng ml^{-1} will have a reasonable chance to fail the test. The sensitivity however differs slightly for endotoxins of other bacteria. Even in the worst performance of the kieselguhr filter a twenty-fold reduction of this high endotoxin content was reached; this reduction further improved during the filtration.

The charcoal filter proved to be very ineffective when water was used as test solution. This might be due to the very loose structure of the filter-material; in the pyrogen-retention experiments no pressurized air was necessary to reach the desired flow. This flow however, which is equivalent to 375 litre m^{-2} h^{-1}, is far beyond the maximum advised by the manufacturer (200 litre m^{-2} h^{-1}). Charcoal has proved to possess a high binding capacity for endotoxins (Nolan et al 1975; Koppensteiner et al 1976). This was demonstrated when endotoxin-containing solutions were mixed with charcoal and shaken for a given time. A reasonable pyrogen retention may be achieved when the flow is reduced. However, these filters do not seem to be suitable for large-scale preparations.

Woog et al (1977) have also investigated the efficiency of kieselguhr and kaolin filters but this was done semi-quantitatively. They challenged the filters with a mixture of bacteria and endotoxins, both of which were added to the test solution. Cultures of *Pseudomonas diminuta* were used to reach a final colony-count of 10^5 ml^{-1}. Cultures of bacteria, whether in the log-phase or in the stationary phase contain considerable amounts of endotoxin (Sullivan et al 1976), which have not been taken into account in their study, so the real endotoxin load is unknown. Furthermore, colony counts of 10^5 ml^{-1} are unrealistic; we never found more than 10 bacteria g^{-1} raw material in raw materials used for the production of infusion fluids (Baggerman 1981). This different experimental design might explain the different results found by Woog et al (1977); in their experiments those filters were less effective than in ours.

Another approach to asbestos-free depth-filtration is formed by the so-called charge-modified-filters. These filters consist of a matrix of cellulose containing diatomite, perlite or sand; the zeta-

potential of these materials being changed from negative to positive by a treatment with a cationic colloid (Fiore & Babineau 1979). These charge-modified depth-filters have also proved to be effective for the removal of pyrogens from water and electrolyte- or carbohydrate-containing solutions (Gerba et al 1980).

As far as water, electrolyte- or carbohydrate-containing solutions are concerned the superiority of asbestos has proved to be a myth. Both the kieselguhr and the kaolin filters provide good alternatives; the latter even seems to be superior to the asbestos filter. Further research is necessary to assess the efficiency of asbestos-free depth-filters for solutions containing amino-acids, plasma substitutes or antibiotics, and to assess the influence of various electrolytes on pyrogen-adsorption.

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