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(54) Title: ISOLATION OF AN ENDOTOXIN INACTIVATOR FROM HUMAN PLASMA (57) Abstract <p>An endotoxin inactivator is isolated from human plasma by anion exchange (DEAE-Sephadex), dye-affinity (Cibacron Blue-Sepharose), and adsorption (on hydroxyapatite) chromatography. The endotoxin inactivator, isolated in essentially pure form, may be used to depyrogenate clinical blood products and culture media.</p>		

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ISOLATION OF AN ENDOTOXIN INACTIVATOR FROM HUMAN PLASMA

5 BACKGROUND AND SUMMARY OF THE INVENTION

The present invention relates the isolation from human plasma of an endotoxin-inactivator which could be added to pyrogenic lots of blood products, such as albumin, immune globulins, antihemophilic factor (AHF) concentrates, Factor IX concentrates, interferons, fibronectin and others, thus rendering them non-pyrogenic, and again suitable for clinical use.

Pyrogenicity in blood products is caused by contamination from endotoxins of Gram-negative bacteria during the manufacturing process. Because of the ubiquitous nature of bacteria, the control of these physiologically active agents is of utmost importance to the plasma fractionation industry, as well as to the entire pharmaceutical industry. The most positive method of control, strict aseptic techniques that limit microbial contamination, cannot, in most cases, maintain complete sterility throughout the manufacturing process. Therefore, manufacturers may at times find their final products pyrogenic at the bulk solution stage prior to filling. The result may be loss of the entire lot, or only partial recovery.

In the plasma fractionation industry the monetary loss due to pyrogenic products may be measured in millions of dollars per year. However, no plasma fractionator has used or reported any means to depyrogenate their endotoxin-contaminated products except that, in the case of depyrogenation of albumin, there are two documented methods.

The first published method for depyrogenation of clinical albumin was reported by Wye and Kim (Vox Sang 32: 182-184, 1977) who mixed pyrogenic albumin with

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5 Cohn ethanol fractions IV-1 and IV-4, based on the findings of Yoshioka and Johnson (J. Immunol. 89: 326-335, 1962), followed by differential thermal heating to recover albumin according to the method of Schneider et al. (Blut 30: 121-134, 1975). The reported method not only requires an excessive amount of Cohn Fractions IV-1 and IV-4, but also suffers considerable losses of
10 albumin. The yield based on 21 batches was about 75%. In some cases, the procedure had to be repeated thus resulting in further losses.

The second method for depyrogenation of albumin was developed by this inventor. It is described in his SN
15 06/635,134, filed July 27, 1984. This method is based on the finding that plasma contains "enzyme(s)" which could detoxify the endotoxins. Pyrogenic albumin was mixed with sufficient plasma to detoxify all endotoxins present. The albumin was then purified to over 99%.
20 (Hao, Vox Sang 36: 313-320, 1979). The resultant albumin had a non-detectable level of endotoxin (less than 0.05 ng/ml) as assayed by Limulus Amebocyte Lysate (LAL) test (Levin et al. J. Lab. Clin. Med. 75: 903, 1970) and confirmed by the USP rabbit pyrogen test.

25 Obviously, the addition of plasma cannot be used for depyrogenation of other clinical plasma products, such as antihemophilic factor (AHF), immune serum globulin (ISG), Factor IX complex concentrate and plasma protein fraction (PPF), since the resulting product
30 would then contain undesired plasma proteins.

It has been known for over 30 years since the first report by Hegemann (Z. Immunitactsforsch 111: 213-225, 1954) that normal human plasma (serum) has the ability to diminish the pyrogenicity of endotoxin derived from
35 the Gram-negative bacteria. This observation was confirmed in subsequent years by many reports (Skarnes et al., J. Exp. Med. 108: 685-700, 1958; Rall et

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5 al., Am. J. Physiol. 188: 559-562, 1957; Rudbach and
Johnson, Nature 202: 811-812, 1964; Yoshioka and
Johnson, J. Immunol. 89: 326-335, 1962; Landy et
al. J. Exp. Med. 110: 731-750, 1959; Skarnes,
Ann. N.Y. Acad. Sci. 133: 644-662, 1966). Landy et
10 al. (1959) and Skarnes (1966) further suggested that the
detoxifying effect by serum (or plasma) is of an
enzymatic nature. Yoshioka and Johnson (1962)
fractionated serum by the Cohn ethanol procedure (Cohn
et al. JACS 68: 459-475, 1946) and found that Cohn
Fraction IV-1 contains the substance(s) which decrease
15 pyrogenicity caused by endotoxins. Skarnes (1966),
using plasma fractions obtained from ion exchange
chromatography, found that the esterase associated with
the alpha-1-lipoprotein appeared responsible for
degradation of endotoxin, and an alpha-1-globulin
20 esterase appeared responsible for inactivation of
endotoxin.

According to Skarnes, "numerous attempts were made
to purify the IV_C fraction in order to separate the
active enzymes. However, neither cellulose nor sephadex
25 columns were well suited to the purpose and although
subfractions were obtained which were rich in either the
lipoprotein esterase or the a₁-globulin esterase, all
such fractions contained both esterases."

Johnson et al. (Amer. J. pathol. 88: 559-574, 1977)
30 later isolated from human serum a single inactivator
which was neither a lipoprotein, nor a serine esterase.
They did, however, find esterase activity associated
with a partially purified inactivator in a sucrose
density gradient system, even though, for unknown
reasons, they did not proceed to isolate this protein in
35 pure form.

Johnson's inactivator "LPS-1" was isolated by a
six-step procedure: fractional precipitation of plasma

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5 with ammonium sulfate at 40-60% saturation; gel
filtration on Sephadex G-150; anion exchange
chromatography on DEAE-cellulose; gel filtration on
10 Sephadex G-200; hydroxylapatite chromatography; and
preparative gel electrophoresis. I found that this
procedure was too long and too tedious. Even though
extreme caution was taken to ensure that every piece of
15 glassware, chemicals, reagents, and every piece of
equipment was pyrogen-free, it was very difficult to
maintain them pyrogen-free since each step required
lengthy dialysis and concentration of the sample.
Therefore, many a time a partially purified fraction was
20 found to have lost its activity at some stage during
purification. There was no way of knowing whether the
loss of activity was due to contamination of endotoxins
or denaturation of the inactivator itself. During
purification, it became obvious that the original
25 procedure called for so many steps mainly for the
removal of a major contaminant, albumin. I found that
bulk of the albumin could be removed at the
DEAE-Sephadex step if stepwise elution at 0.15 M NaCl
concentration was used. By the use of Cibacron-Blue
30 Sepharose (CBS) right after DEAE-Sephadex step, I
removed the remaining albumin. I used the same buffer
system (0.02 M phosphate buffer, pH 7.35) in both steps
so that the DEAE-eluate could be simply diluted 3-fold
or diafiltered prior to application to CBS column.
35 Because of the complete removal of albumin, a highly
purified EI could be readily obtained by hydroxylapatite
chromatography thus eliminating the steps of gel
filtration on G-150 and G-200 and preparative gel
electrophoresis (taught by Johnson) which would have
been a bottleneck if large quantities of EI were to be
prepared.

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Endotoxins are high molecular weight complexes, associated with the outer membrane of Gram-negative bacteria that induce pyrogenic reactions upon intravenous administration. Endotoxins contain lipid, carbohydrate and protein. Purified endotoxins do not contain protein, and therefore, are referred to as lipopolysaccharide (LPS). It has been shown that LPS contains three distinct chemical regions; Region I, O-specific polysaccharide carrying the main serologic specificity, is linked to the core polysaccharide, known as Region II. This core material is linked in turn to the lipid component--Region III or Lipid A. (Westphal, O. Int. Archs. Allergy Appl. Immun. 49: 1-43, 1975 and Bradley, S.G. Ann. Rev. Microbial. 33: 67-94, 1979).

It is the lipid A which is responsible for most, if not all, of the biological activities of endotoxin (Galanos, et al. Eur. J. Biochem. 19: 145-152, 1971; Galanos, et al. Eur. J. Biochem. 22: 218-224, 1971; Luderitz, et al. The Chemistry, Biology and Clinical Significance of Endotoxins Univ. of Chicago Press, pp. 9-21, 1973; Rietschel et al. Infect. Immunity 8: 173-177, 1973). For example, when free lipid A is complexed with bovine serum albumin, or human serum albumin, pyrogenicity is induced comparable to that of intact endotoxin according to rabbit pyrogen test (Galanos et al. 1972; and Rietschel et al. 1973). Furthermore, the activity of Lipid A derived from E. coli and various strains of Salmonella are similar and the pattern of febrile response is identical to that produced by intact endotoxin (Luderitz et al. 1973).

Lipid A is composed of repeated disaccharides of glucosamine, which is highly substituted with ester-linked long chain fatty acids (Westphal, 1975 and Luderitz et al. Int'l Sympo. on Pyrogen, Univ. College, London pp. 10-19, 1975). Furthermore, it was found that

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Lipid A is linked to core heteropolysaccharides by
2-keto-3-deoxyoctonic acid (KDO) which is unique to
bacterial liposaccharides but does not contribute to
endotoxicity (Rietschel et al. 1973). It is the
ester-linked fatty acids which are responsible for the
biological activity. Removal of fatty acid residue
abrogates the biological activity of Lipid A (Westphal,
1975, Bradley 1979, and Luderitz, 1975), but the
remainder of the Lipid A molecule may determine
solubility, conformation, distribution within the body
and affinity for receptor site (Bradley 1979). Based on
information described above, it may be concluded that
human plasma contains an endotoxin-inactivator, which is
most probably an esterase that detoxifies Lipid A by
breaking off the ester-linked fatty acids.

The present invention for the isolation of an
endotoxin-inactivator from human plasma involves three
purification steps:

- (1) Adsorption of plasma on DEAE-Sephadex followed by
stepwise elution
- (2) Adsorption of DEAE eluate on Cibacron-Blue
sepharose (CBS) followed by stepwise elution, and
- (3) Adsorption of the CBS elute on hydroxylapatite
followed by stepwise elution

The final product is homogeneous as judged by
polyacrylamide gel electrophoresis (PAGE),
sodium-dodecyl sulfate PAGE (SDS-PAGE) and
immunoelectrophoresis. The molecular weight of this
protein is estimated as falling between 61,000 and
65,000 as judged by SDS-PAGE and high performance liquid
chromatography (HPLC).

The purified inactivator has been found to
inactivate all three types of lipopolysaccharides
tested; they are S. typhosa, S. enteritides and E. coli
strain 055: 85. It has also been found that highly

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pyrogenic albumin can be made non-pyrogenic by mixing it with titrated amount of this inactivator. The inactivation of endotoxin was evidenced by the Limulus amebocyte lysate test in vitro and was further confirmed by the USP rabbit pyrogen test in vivo.

Chibata, US 4,381,239 reviews methods of removing pyrogen: (1) adsorption; (2) decomposition with acid or alkali; (3) decomposition with an oxidizing agent; or (4) filtration. Chibata taught that bacterial LPS is selectively adsorbed by a heterocyclic nitrogen compound.

The filtration of pyrogens from biological fluids is known. See Hou, US 4,488,969; Grabner, US 3,897,309; Chibata, US 4,160,697; Nakamura, US 4,259,448.

Mannuzza, US 4,380,511 teaches the removal of pyrogens from blood protein products by absorbing the blood protein on blue dextran, washing away the pyrogen with a low ionic strength solution, and desorbing the column with a high ionic strength solution.

Babb, US 4,381,004 speaks of a "microorganism deactivator" for extracorporeal treatment of blood. However, this deactivator is an antimicrobial agent, not an inactivator of endotoxin. Babb provides for adsorption, rather than inactivation, of bacterial endotoxins.

Shanbrom, US 4,069,216 discloses the "reworking" of pyrogenic lots of Factor VIII by a one or two step cold 6% PEG precipitation. The pyrogens are washed away from the Factor VIII precipitate. They are not inactivated. See also Shanbrom, US 4,188,318.

Cano, US 4,000,257 teaches use of ethyl or butyl acetate to extract endotoxins from an influenza virus vaccine to obtain a vaccine of low pyrogenicity.

Smith, US 3,659,027 discloses destruction of pyrogens in water intended for parenteral use by strong

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alkali. Clearly, such harsh treatment is unsuitable for protein preparations.

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Dasinger, US 3,644,175 describes the inactivation (by acidification and heating) of endotoxins of gram-negative bacteria intended for use as a protein source.

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Akcasu, US 4,070,289 depyrogenates water by distillation under pressure.

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GB 1,418,286 teaches the removal of pyrogens from urokinase (a product of human urine) by retaining the pyrogens on an anion exchange cellulose, such as diethylamino ethyl (DEAE) cellulose.

GB 1,557,545 teaches that urokinase can be reversibly absorbed on a hydrophilic polysaccharide which does not retain pyrogens.

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BRIEF DESCRIPTION OF THE DRAWINGS

- 5 Fig. 1 Elution profile from DEAE-Sephadex chromatography (step 1).
- Fig. 2 Elution profile from Cibacron-Blue Sepharose chromatography (step 2).
- Fig. 3 Elution profile from hydroxylapatite chromatography (step 3).
- 10 Fig. 4 Immuno-electropherogram of endotoxin inactivator.
- Fig. 5 Polyacrylamide gel slab electropherogram of endotoxin inactivator.
- 15 Fig. 6 SDS-PAGE analysis of endotoxin inactivator.
- Fig. 7 HPLC elution profile of endotoxin inactivator mixed with pyrogenic albumin.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 In the method of the present invention for isolation of this endotoxin-inactivator (EI), it was carried out in a series of steps which are described hereinafter as Example 1.

10 The starting material can be fresh, fresh frozen, liquid, outdated plasma, cryo supernatant (after cryo precipitate is removed) or serum.

EXAMPLE 1

15 In essence, endotoxin-inactivator can be purified from plasma by three steps: namely, adsorption on DEAE-Sephadex and stepwise elution to remove the bulk of plasma proteins; adsorption on Cibacron-Blue-Sepharose and stepwise elution to remove the main contaminant, albumin; and adsorption on hydroxylapatite and stepwise elution to obtain a highly purified
20 endotoxin-inactivator.

(1) DEAE-Sephadex Chromatography

Figure 1 is the elution profile of protein distribution from DEAE-Sephadex. Dialyzed plasma, 50 ml, was applied to a column, 1.8 x 20 cm. Stepwise
25 elution using 0.07 M, 0.15 M and 0.25 M NaCl was then carried out. Each tube collected 15 ml eluate, and every second tube was assayed for activity. Buffer (0.02 M Phosphate, pH 7.35) containing 0.07 M NaCl eluted the residual reddish-colored fraction
30 (transferrin) and a yellow-colored fraction, albumin. Buffer containing 0.25 M NaCl eluted one protein peak with the inactivator activity concentrated in the ascending part of peak, and a slightly bluish fraction, which is ceruloplasmin, in the descending part of the
35 protein peak. Fractions with inactivator activity were pooled according to their endotoxin inactivating activity as assayed by the LAL test system.

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While DEAE-Sephadex Chromatography is preferred, those skilled in the art will recognize that other anion exchange chromatography techniques might prove fruitful. Elution at 0.25 M NaCl is preferred, but salt concentrations ranging from 0.2-0.3 M may prove useful.

(2) Cibacron-Blue Sepharose Chromatography

Figure 2 is the elution profile of protein distribution from Cibacron-Blue Sepharose. Diafiltered DEAE eluate containing EI was applied to the CBS column (1.25 x 20 cm) previously equilibrated with 0.02 M phosphate buffer, pH 7.35. Stepwise elution using 0.07 M, 0.2 M and 2 M NaCl was then carried out. Each tube collected 10 ml eluate, and every second tube was assayed for activity. At 0.07 M NaCl concentration, an inactive protein peak was eluted. At 0.20 M NaCl concentration, a second protein peak with inactivator activity was eluted. At 2.0 M NaCl concentration, a third protein peak, mostly albumin, was eluted. It should be noted that even though each of these peaks looked more or less symmetrical, it by no means represented only one protein.

It may be possible to substitute other dye affinity chromatographic separations for the preferred Cibacron-Blue Sepharose chromatographic step described herein. While elution at 0.2 M NaCl is preferred, it is believed that a range of salt concentrations such as 0.15-0.25 M will be efficacious.

(3) Hydroxylapatite Chromatography

Figure 3 is the elution profile of protein distribution from hydroxylapatite chromatography. Diafiltered CBS eluate was applied to a HTP column (1 x 9.5 cm) previously equilibrated in 0.02 M phosphate buffer pH 6.8. In order to obtain good flow rate 2 g of Sephadex G-25 was added to 10 g of HTP. Stepwise elution using 0.14 M and 0.3 M phosphate buffer, pH 6.8

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was carried out. Tubes of 7 ml were collected. Fractions containing EI activity was eluted at 0.14 M phosphate concentration at pH 6.8. Another protein peak was eluted at 0.3 M phosphate concentration at the same pH.

It will be understood that those skilled in the art may be able to effect a further purification using another adsorption medium in place of hydroxylapatite. While elution at 0.14 M phosphate is preferred, those skilled in the art may wish to employ concentrations of 0.1-0.25 M phosphate.

(4) Yield, Recovery and EI Concentration

Based on the results of 5 runs each starting from 50 ml of fresh frozen plasma derived from 400 liter plasma pool an average yield of 1.4 mg of EI was obtained as assayed by Lowry (J. Biol. Chem. 193: 265, 1951). Since there was no report in the literature stating the concentration of EI in plasma, the percentage of recovery and concentration of EI are calculated as follows. Based on the activity assay, every 0.05 ml plasma inactivates 31.25 ng endotoxin standard (E. coli 055: B5). Therefore, 50 ml plasma would inactivate a total of 31,250 ng. It has also been found that every 0.05 ml EI preparation containing 0.014 mg EI would inactivate 2,560 ng which represents 8.2% recovery and the concentration of EI in plasma could therefore be calculated to contain $(31,250 \text{ ng} / 2,560 \text{ ng}) \times 1.4 \text{ mg} = 17 \text{ mg}$ for every 50 ml or 34 mg/100 ml.

CHARACTERIZATION OF EI

(1) Purity and molecular weight determinations

The purified EI was subjected to immunoelectrophoresis (IEP), polyacrylamide gel slab electrophoresis (PAGE), SDS-polyacrylamide gel slab electrophoresis (SDS-PAGE) and high performance liquid chromatography (HPLC).

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Figure 4 is the IEP result of the purified EI which shows one precipitation line when reacted with anti-whole serum. The conditions of the immunoelectrophoresis of EI were as follows: Top Well, EI, 7 ug; Bottom Well, Plasma (1:2), 5 ug; and Trough, anti whole serum 100 ul.

Figure 5 is the PAGE result of EI which shows one protein band (5A), midway between albumin and transferrin, when compared to that of plasma (5B). The conditions of the Polyacrylamide gel slab electrophoresis were as follows: Running gel 7.5% and stacking gel 3.5%; Endotoxin inactivator, 6 ug; and Plasma (1: 17), 5 ug.

Figure 6 is the result of SDS-PAGE, which again shows one protein band (6B) having a molecular weight of approximately 61,000 when calculated from the protein standards (6A). Protein standards (6A) are (from top down): myosin (H chain), 212,000; phosphorylase B, 97,500; bovine serum albumin, 68,000; ovalbumin, 43,000; alpha-chymotrypsinogen, 25,700 and beta-lactoglobulin, 18,400. EI (6B): 24 ug.

Figure 7 is the HPLC result when EI is mixed with pyrogenic albumin. Based on these findings the molecular weight of EI is the same as albumin (HSA) which is 65,000. The test sample was made up from equal volume of human serum albumin (HSA), 0.15%, and EI, 0.12% in 0.05 M phosphate, pH 7.0, containing 0.15 M NaCl. The column used was TSK 3000, 7.5 x 300 mm.

(2) EI Activity

Lipopolysaccharide (LPS) derived from Salmonella typhosa, Salmonella enteritides and Escherichia coli 0.55: B5 of 2-fold serial dilutions were mixed with EI and the endotoxins remained in the mixture were assayed by LAL with the sensitivity of the lysate being 0.05 ng/ml. The LAL assay is based on the discovery by Bang,

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Johns Hopkins Hosp., 98: 325 (1956) of the gelation of a lysate of amebocyte of Limulus, the horseshoe crab. The results are shown in Table 1. The last column of the table is the total nanogram of LPS inactivated per mg of EI calculated from the results in the reaction mixture (middle column).

TABLE 1: INACTIVATION OF VARIOUS LPS BY ENDOTOXIN
INACTIVATOR

<u>LPS</u>	<u>NG INACTIVATED</u>	
	<u>IN 0.5 ML</u>	<u>NG INACTIVATED</u>
	0.014 MG EI	MG EI
S. Typhosa	51.2-102.4 ng	3,657-7,314 ng
S. enteritides	12.8-25.6 ng	914-1,829 ng
E. coli 055:B5	12.8-25.6 ng	914-1,829 ng

Because of the nature of the LAL assay in which LPS solutions were prepared by 2-fold serial dilutions, the EI activity toward each LPS was expressed in a range for the given amount of EI. Although it has been known that LPS derived from one type of bacteria could be as much as 6 times more potent than the other (Greisman and Hormick, Proc. Soc. Exp. Biol. Med. 131: 1154, 1969), the purity of each LPS used in these experiments were not necessarily the same thus resulting, at least in part, in the different ranges of inactivation.

(3) Rabbit Pyrogen Test

In order to ascertain that the inactivation was not a result of inhibition of the LAL test but as a result of the detoxification of the endotoxins, an aliquot of a highly pyrogenic 25% albumin solution having an endotoxin level of 12.8 ng/ml was divided into two vials, 25 ml each. To one vial, 0.125 mg of EI in 0.89 ml of 0.02 M phosphate, pH 7.35, was added, and to the second vial (Control) 0.89 ml of plain buffer was

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5 added. Both vials were then incubated at 37 deg. C for
15 min. and rabbit pyrogen tests (3 ml/kg) were carried
out. The results showed that the Control, which was not
treated with EI, induced an aggregate temperature rise
of 2.1 deg. C (0.2 , 0.9 , 1.2 deg. C) whereas the
10 EI-treated albumin induced an aggregate temperature rise
of 0.4 deg. C (0.0, 0.0, 0.4 deg. C), clearly suggesting
that the endotoxin had been detoxified. (Any parenteral
intended for clinical use has to pass the rabbit pyrogen
test with an induced temperature rise not greater than a
total of 1.5 deg. C for three rabbits, and none of the
15 three rabbits may have a temperature rise of more than
0.5 deg. C). The term "essentially nonpyrogenic", as
used herein, means a preparation which passes the USP
rabbit pyrogen test.

The term "endotoxin inactivator" encompasses
substances which merely reduce the pyrogenic activity of
20 an endotoxin sufficiently to render an endotoxin
containing composition "essentially nonpyrogenic," i.e.,
acceptable for use, particularly pharmaceutical use. To
"depyrogenate" is to reduce the pyrogenic activity of a
composition.

25 (4) Stability of EI

Preliminary results indicate that the activity of
EI prepared by this procedure appears to be stable for
up to 48 hours at +5 C. Whereas, samples kept at -20 C
and -80 C remained active even after one month.

30 COPURIFICATION OF ENDOTOXIN INACTIVATOR WITH OTHER BLOOD PROTEINS

Products such as AHF and Factor IX complex are
routinely isolated prior to ethanol fractionation. If
35 there is a need of EI, it appears feasible to
incorporate the present procedure into the scheme when
Factor IX complex is isolated, since the procedure also

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involves the use of DEAE-Sephadex. (Heystek, et al.,
Vox Sang, 25: 113, 1973.) After DEAE-Sephadex
5 adsorption and prior to elution of Factor IX complex at
2.0 M NaCl, a wash step using 0.15 M NaCl elutes most of
the albumin which is to be added to the starting plasma
for recovery of albumin. An additional stepwise elution
of the DEAE-cake at 0.25 M NaCl would yield a partially
10 purified EI which could be further purified without
interfering with the recovery of either albumin or
Factor IX complex.

As described above, the first step of the
purification procedure was to adsorb dialyzed plasma on
15 DEAE-Sephadex followed by stepwise elution at different
ionic strength. Partially purified EI was eluted at
0.25 M NaCl in 0.02 M phosphate buffer, pH 7.35. This
step, with modification, could conceivably be
incorporated into the procedure for isolation of Factor
20 IX complex. One of the widely used procedures, Suomela,
et al., Vox Sang, 33:37 (1977), for isolation of Factor
IX complex (II, VII, IX and X) consists of adding
DEAE-Sephadex (1.5 g/L plasma) to plasma or cryo
supernatant, washing DEAE cake with 0.01 M sodium
25 citrate buffer containing 0.15-0.17 M NaCl, pH 7.0 and
eluting Factor IX complex with 0.03 M sodium citrate
containing 2 M NaCl. The present method for isolation
of EI requiring 0.25 M NaCl in 0.02 M phosphate buffer
could be readily incorporated in between the washing and
30 eluting steps. It is expected that the introduction of
an additional elution step for EI would result in an
insignificant reduction in the overall yield of Factor
IX due to partial elution of Factor VII at this ionic
strength, but the specific activity of the final
35 products in terms of Factor IX should be higher since
Factor IX remains tightly bound under such conditions.

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DEPYROGENATION OF OTHER PRODUCTS

5 The endotoxin inactivator (EI) may also be used to inactivate other products in addition to those derived from plasma. Furthermore, the endotoxin inactivator does not have to be in pure form to be of commercial significance; partially purified EI or even whole plasma might well be suitable in many situations.

10 Testing revealed that six commercially available culture media were all pyrogenic; all six had between 2.5 to 10 endotoxin units (EU) per ml of culture media. Such level of endotoxin would potentially have an adverse effect on the cells to be cultured in the said media. However, all six media could be made non-pyrogenic by adding a small amount of plasma or a partially purified EI as shown in the following table.

TISSUE CULTURE MEDIUM, 500 ml, RPM I 1640

20 <u>Manufacturer</u>	<u>Endotoxin Units,</u> <u>EU/ml</u>	<u>Amount of Plasma</u> <u>Needed to</u> <u>Depyrogenate</u> <u>ml</u>	<u>Amount of EI*</u> <u>Needed to</u> <u>Depyrogenate</u> <u>ml</u>
1. KC Biological	5 - 10	3.0	3.0
2. Hazleton	5 - 10	3.0	3.0
3. Gibco	5	1.5	1.5
4. M. A. Bioproducts	5	1.5	1.5
5. S & S Media	2.5	0.75	0.75
6. Quality Biological	2.5	0.75	0.75

(*partially purified EI, partially concentrated from eluting buffer, having same total activity as equal volume of plasma)

35 Fetal calf serum, which is commonly used to supplement tissue culture media, failed to exhibit any endotoxin-inactivating activity.

For veterinary products, it is generally desirable to purify the EI from the plasma of the same species of animal

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as that to be treated. In this way, use of a partially purified EI would not introduce foreign proteins.

5 It has also been found that bovine plasma has the same level of inactivating activity as human plasma. However, equine plasma has been found to contain only 25% of the activity of those of human and bovine plasma.

10 Furthermore, plasma and endotoxin inactivator stored in lyophilized form and at +4°, -20°, and -70°C have been found to retain all their activity even after five weeks of storage.

15 It is believed that the endotoxin inactivator of the present invention could be useful in depyrogenating any product where pyrogens are of concern.

20 It will be apparent that various changes may be made in the method as described herein without departing from the spirit and scope of the invention or sacrificing its material advantages, the forms hereinbefore described being merely preferred embodiments thereof.

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I claim:

5 1. A method for the isolation, from an endotoxin inactivator-containing fraction of blood, of an endotoxin inactivator in essentially pure form which comprises

10 (a) ion-exchange chromatography of an endotoxin inactivator-containing fraction of blood;

(b) dye-affinity chromatography of the product of step (a); and

(c) adsorption chromatography of the product of step (b).

15 2. The method of claim 1 in which the ion-exchange chromatography is DEAE-Sephadex chromatography.

20 3. The method of claim 1 in which the dye-affinity chromatography is CIBACRON-BLUE Sepharose chromatography.

4. The method of claim 1 in which the adsorption chromatography is on hydroxylapatite.

25 5. A method for the isolation from plasma or serum of an endotoxin inactivator in essentially pure form, which comprises

(a) DEAE-Sephadex chromatography of plasma or serum;

30 (b) CIBACRON-BLUE Sepharose chromatography of the product of step (a); and

(c) hydroxylapatite chromatography of the product of step (b).

35 6. The endotoxin inactivator isolated by the method of claim 5.

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- 5 7. Essentially pure endotoxin inactivator, eluting at about 0.2-0.3 NaCl from a DEAE-Sephadex adsorbent, about 0.15-0.25 M NaCl from a Cibacron-Blue Sepharose adsorbent, and at about 0.1-0.25 M phosphate from a hydroxylapatite adsorbent.
- 10 8. Essentially pure Endotoxin inactivator, as a protein having a molecular weight of about 61,000 daltons as determined by SDS-PAGE with bovine serum albumin and ovalbumin as standards, and eluting at 9.79 minutes on a TSK3000 HPLC column, 7.5 x 300 mm, in a 0.05 M phosphate buffer (pH 7.0) containing 0.15 M NaCl.
- 15 9. Essentially pure Endotoxin inactivator, capable of inactivating at least 3,657 ng of S. typhosa endotoxin, 3,657 ng of S. enterides endotoxin, and 914 ng of E. coli 0.55: B5 endotoxin, for each mg of inactivator.
- 20 10. A homogeneous serum protein preparation intended for parenteral administration which is essentially nonpyrogenic.
- 25 11. A homogeneous plasma protein composition wherein the plasma protein is a protein other than albumin, and wherein all original endotoxins have been enzymatically inactivated.
- 30 12. A method of depyrogenating a pyrogenic blood product which comprises the addition to the blood product of an endotoxin inactivator in essentially pure form.
- 35 13. The method of claim 12 where the endotoxin inactivator is isolated by the method of claim 5.

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5 14. The method of claim 2, in which the endotoxin inactivator is eluted by increasing the ionic strength of an eluting buffer.

10 15. The method of claim 2, in which the DEAE-Sephadex chromatography comprises contacting plasma with DEAE-Sephadex in the presence of an equilibrating buffer whose ionic strength does not exceed 0.15 and whose pH is 6-9, and eluting the adsorbed material with a buffer whose ionic strength is greater than 0.15 M and whose pH is 6-9.

15 16. The method of claim 15 in which the eluate from the DEAE-Sephadex chromatography is concentrated and diafiltered so that its ionic strength is 0.07-0.15 and its pH is 6-9.

20 17. The method of claim 3 in which material adsorbed on the CIBACRON-BLUE adsorbent is eluted by a buffer having an ionic strength of 0.1-0.3 M and a pH of 6-9.

25 18. The method of claim 17 in which the CIBACON-BLUE eluate is concentrated and diafiltered.

30 19. The method of claim 4 in which the essentially pure endotoxin inactivator is eluted by a phosphate buffer of at least 0.14 M but less than 0.3 M phosphate concentration, at a pH of 6-7.

20. The composition of claim 11 where the protein is albumin.

35 21. The composition of claim 11 where the protein is an immunoglobulin.

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22. The composition of claim 11 where the protein is antihemophilic factor.

23. The composition of claim 11 where the protein is Factor IX.

24. The composition of claim 11 where the protein is an interferon.

25. A method of separating an endotoxin inactivator from human serum albumin which comprises DEAE-Sephadex chromatography with elution at about 0.15-0.25 M NaCl, followed by CIBACRON-BLUE Sepharose Chromatography.

26. A method of reducing the pyrogenicity of a pyrogenic culture medium which comprises the addition to the culture medium of an endotoxin inactivator.

27. The method of claim 26 in which the endotoxin inactivator is provided in essentially pure form.

28. The method of claim 26 in which the culture medium is adapted to culture animal cells, and the endotoxin inactivator is derived from the plasma of that species of animal.

29. A method of copurifying Factor IX complex and endotoxin inactivator which comprises ion exchange chromatography, wherein the Factor IX complex and the endotoxin inactivator are separately eluted by buffers of different ionic strength.

30. The method of claim 29 in which the chromatography employs DEAE as the ion exchanger.

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31. The method of claim 30 in which the endotoxin
inactivator is eluted at about 0.25M salt and the
Factor IX complex at about 2M salt.

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FIG. 1.

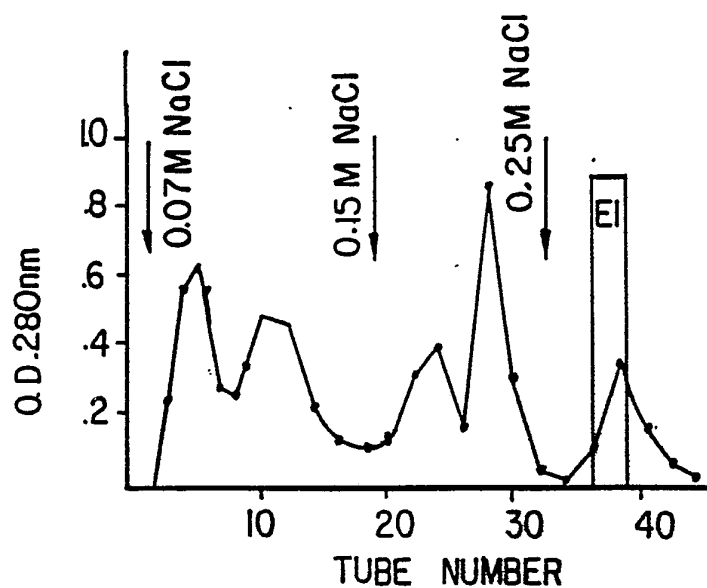
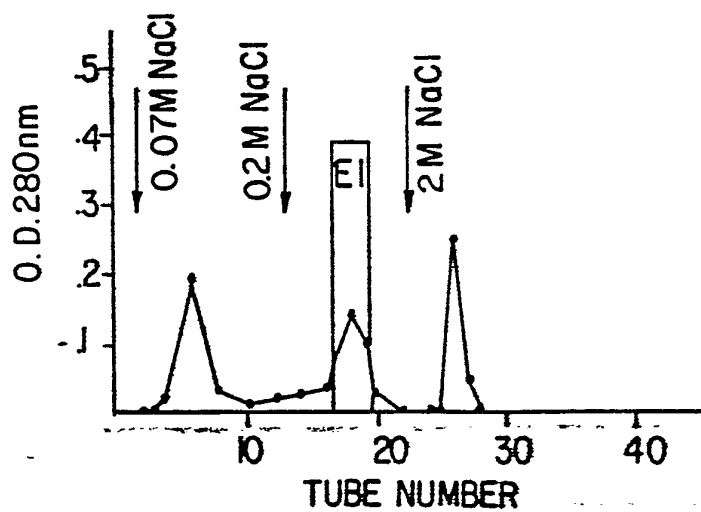


FIG. 2.



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FIG. 4



FIG. 5



A B

FIG. 6



A B

FIG. 3.

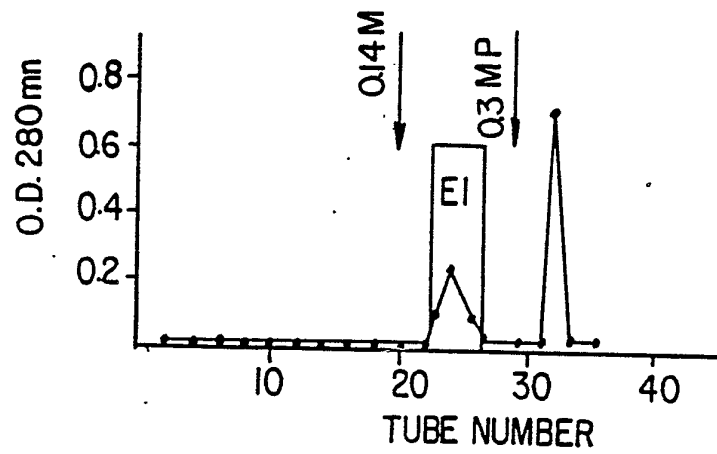
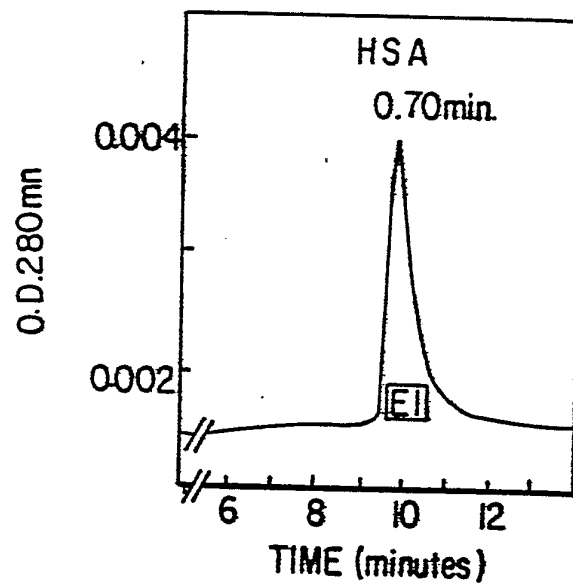


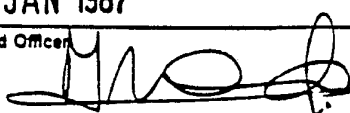
FIG. 7.



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 86/01640

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
⁴ A 61 K 35/14; A 61 K 37/02; A 61 K 39/395; A 61 K 45/02; IPC : A 61 L 2/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁴	A 61 L A 61 L	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	Chemical Abstracts, volume 82, no. 9, 3 March 1975, (Columbus, Ohio, US), R.H. Luff et al.: "Inactivation of endotoxin by rabbit plasma containing endogenous pyrogen", see page 431, abstract no. 55874m, & IRCS Libr. Compend. 1973, 1(2), 17.1.5	1
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A	US, A, 4069216 (EDWARD SHANBROM) 17 January 1978 (cited in the application)	
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
9th December 1986	21 JAN 1987	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	M. VAN MOL 	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/US 86/01640 (SA 14278)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/12/86

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For more details about this annex :
see Official Journal of the European Patent Office, No. 12/82

INTERNATIONAL APPLICATION NO.

PCT/US 86/01640 (SA 14278)

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US-A- 4381004	26/04/83	None		
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