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(57) Abstract

Water-soluble polymyxin B/dextran conjugates (or salts thereof) having anti-endotoxin activity greater than unconjugated polymyxin B. They can be prepared by reacting polymyxin B or a salt thereof with dextran in an aqueous medium at a pH of from about 9.3 to about 10 and a temperature of from about 30° to about 35 °C.

POLYMYXIN CONJUGATES

Summary of the invention

The present invention relates to a process for producing water-soluble conjugates (or salts thereof) of polymyxin B (PMB) or salts thereof and dextran which comprises reacting polymyxin B or a salt thereof with dextran in an aqueous medium at a pH of from about 9.3 to about 10, preferably from about 9.5 to about 9.7, and a temperature of from about 30° to about 35°C, preferably about 32°C. A preferred process variant involves reacting polymyxin B sulfate with oxidized dextran at a temperature of about 32°C to covalently attach the polymyxin B molecule to the dextran through an amine bond. The improved conjugates (or salts thereof) prepared by the process of the present invention are easily and consistently reproducible and exhibit significantly improved pharmacological properties, such as higher potency and anti-endotoxin activity greater than the anti-endotoxin activity of unconjugated polymyxin B.

The invention also includes water-soluble conjugates (or salts thereof) comprising polymyxin B or a salt thereof and dextran, the conjugates (or salts thereof) having anti-endotoxin activity greater than the anti-endotoxin activity of unconjugated polymyxin B.

Background of the invention

The medical need for an effective therapy for systemic inflammatory response syndrome (SIRS) and septic shock is widely recognized and the patient base is large, with over 100'000 critical cases of SIRS/sepsis in developed nations requiring acute therapy and nearly 1 million patients at high risk for sepsis who could benefit from prophylaxis.

Endotoxin is recognized as the major initiator/primary mediator in the pathogenesis of SIRS/sepsis based upon a large body of human and animal studies, thus providing a clear scientific rationale for an anti-endotoxin approach to sepsis. Endotoxins or lipopolysaccharides are structural molecules derived from the cell walls of Gram-negative bacteria. When introduced into the bloodstream, they can interfere with the regulation of body temperature and cause fever. They also have a toxic effect, leading to cardiac, pulmonary and kidney failure. Endotoxin-related diseases are a leading cause of death among those patients in intensive care units.

Unique among antibiotics is the ability of PMB to neutralize endotoxin, accomplished by binding to the lipid A region of the endotoxin molecule. PMB from Bacillus polymyxa (B. aerosporus) is a highly charged amphiphilic cyclic peptidolipid. It is also useful in combating various fungal infections, especially those arising in immunocompromised individuals. However, PMB has some properties which renders it less than an ideal antibiotic. First, it has a short half-life in the body, requiring repeated dosages in order to be effective. Secondly, as it passes through the kidney it can cause extensive damage. Thirdly, at high doses it possesses neurotoxic properties which cause respiratory paralysis.

Previously, researchers have conjugated PMB to immobile or fixed molecules, see e.g. Issekutz, J. Immunol. Methods 61 (1983) 275-281, describing the binding of PMB to Sepharose. These conjugates, while useful in purification techniques, are not suitable for in vivo therapeutic use.

One approach to achieve pharmacological activity, increased duration, or decreased organ toxicity has involved the conjugation of drugs to large molecular weight macromolecules such as dextran, polyethylene glycol or polyvinylpyrrolidine. Attempts in this area of polymer conjugation have been met with only limited success, however. For example, the conjugated form of procainamide (an antiarrythmic drug) was less active and exhibited a shorter half life than native procainamide (Schact et al., Ann N.Y., Acad. Sci. 416 [1985] 199-211). Similarly, the prostaglandin analog B245, linked to a carrier, was less effective (by several orders of magnitude) than the native molecule (Bamford et al., Bioch.Biophys.Acta 886 [1986] 109-118). Reductions in biological potency have also been described for conjugated forms of kallikrein, aprotinin, bradykinin (Odya et al., Biochem.Pharmacol. 27 [1978] 173-179), the anti-tumor drugs daunorubicin (Hurwitz et al., J.Appl.Biochem. 2 [1980] 25-35), and mitomycin C (Takakura et al., Cancer Res. 44 [1984] 2505-2510). Conjugated enzymes also suffer a reduction in biological activity due to steric hindrance and reduced substrate accessibility (Blomhoff et al., Biochem.Biophys.Acta 757 [1983] 202-208; Marshall et al., J.Biol.Chem. 251(4) [1976] 1081-1087; R.L. Foster, Experimentia 31(7) [1975] 772-773; Wileman et al., J.Pharm.Pharmacol. 35 [1983] 762-765). There are however, some examples of improvements in circulatory half-life after conjugation (Wileman, supra; Kaneo, Chem.Pharm.Bull. 37(1) [1989] 218-220).

It would be desirable to develop a form of PMB which would stay in the blood stream longer, and/or does not have neuro- or nephrotoxicity at therapeutic doses. USP 5'177'059 and its equivalents in other countries, such as EP 428486, describe polymyxin B conjugates with polysaccharides such as dextran or hydroxy ethyl starch, proteins such as albumin, and polymers such as polyvinylpyrrolidone, polyethylene glycol and polyvinyl alcohol. That patent specifically describes a process for chemical conjugation of polymyxin B to dextran by reacting the materials at ambient temperature (i.e. about 25°C or less) and pH 8.5-9.0. The conjugates described in that patent are less toxic than unconjugated polymyxin B and have anti-endotoxin activity which is not clearly defined but appears to be less than or equal to that of unconjugated polymyxin B.

The process of the present invention is an improvement over the process described in that patent, and provides conjugates (or salts thereof) of polymyxin B or a salt thereof and dextran which have, surprisingly, improved characteristics as compared to known PMB - dextran conjugates, in particular greater anti-endotoxin activity than unconjugated polymyxin B, higher potency, better reproducibility and improved pharmacological properties. Particularly surprising is that the novel PMB - dextran conjugates (or salts thereof) of the present invention have consistently and reproducibly greater anti-endotoxin activity than unconjugated PMB. While the molecular basis for this unexpected novel characteristics is not clearly understood, it is likely that at the reaction conditions which are used with the present invention in the preparation of the conjugates the pH change alters the distribution of protons on the basic sidechains. At higher pH one or more sidechain amino groups may become more available for attachment to the dextran via reductive amination. Insasmuch as each sidechain amino group will have a characteristic pKa, the higher pH will liberate the same amine each time. If that amine affords a particularly favourable attachment (from the standpoint of potency of the conjugate), then a better form of conjugate emerges, because that particular attachment route contributes more to the overall structure. This structure confers upon the conjugates in a consistently reproducible manner a level of activity which is above the level of activity of native PMB, e.g. 125 % or more, of the activity of native PMB.

Detailed description

The present invention involves an improved process for producing a water-soluble conjugate (or salt thereof) of polymyxin B and dextran which is useful in treating fungal and bacterial infections and preventing disease caused by bacterial endotoxin. Specifically, the present invention relates to a process for producing a water-soluble polymyxin B / dextran conjugate (or salt thereof) comprising the reaction of polymyxin B or a salt thereof with dextran in an aqueous medium at a pH of from about 9.3 to about 10 and a temperature of from about 30° to about 35°C. PMB is a peptide antibiotic and approved pharmaceutical agent with modest antibiotic activity. It has been in clinical use for over 40 years for topical and parenteral applications. Development of bacterial resistance to PMB is very rare. PMB binds endotoxin with an affinity of about 10^{-6} M and can neutralize the biological effects of endotoxin from all clinically important gram-negative bacteria in a large number of in vitro and in vivo models. In vitro, it has been used as a standard to neutralize endotoxin. Moreover, in vivo, PMB protects against pathology of bacterial sepsis in many animal models such as acidosis and hypotension in enterobacterial infections and lethality due to gram-negative sepsis in dogs, rabbit, rats and mice. It also has potential in preventing sepsis in humans such as burn patients.

The PMB used for the preparation of the conjugate of the present invention is available commercially. PMB can be prepared by fermentation of Bacillus polymyxa (Prazmowski) Migula. PMB consists of a mixture of several related decapeptides. PMB or a pharmaceutically acceptable salt thereof is useful in this invention (such as polymyxin B sulfate and the like).

The dextran used in the process of the present invention can be any of the conventional pharmaceutically acceptable dextrans. Preferably the dextran is chemically modified to covalently bind to peptides, such as oxidized by an oxidizing agent, e.g. sodium periodate. The dextran should preferably have a weight average molecular weight of from about 25'000 to about 500'000; more preferably from about 50'000 to about 200'000; more preferably from about 63'000 to about 76'000. The molecular weight can be determined e.g. by gel permeation high performance liquid chromatography. The most preferred dextran is prepared by fermentation of Leuconostoc mesenteroides (NRRL B-512). It is comprised of

glucose units which are α[1-6]-linked in a long linear chain with approximately 5% α[1-3] branching. Of the branched chains, about 85% have 1 to 2 glucose units and the remaining 15% an average of 33 units.

The general procedures for preparing conjugates of PMB and dextran in aqueous media are described in USP 5'177'059 and are applicable to the present invention. In a preferred embodiment of the present invention, partially oxidized dextran is prepared by reaction with an oxidizing agent such as sodium periodate ($NaIO_4$). This treatment creates aldehydes via oxidative cleavage of vicinal diols on the glucose monomers. Upon exposure of the partially oxidized dextran to PMB or a salt thereof, Schiff's bases are formed. The production of conjugates within the scope of the present invention requires careful control of the pH at from about 9.3 to about 10, preferably from about 9.5 to about 10, more preferably from about 9.5 to about 9.7, during the reaction of PMB and dextran, especially during the Schiff's base formation stage. The pH is preferably maintained using a borate buffer, especially one comprising sodium tetraborate. Also, the temperature of the aqueous medium is maintained at from about 30° to about 35°C, more preferably about 32°C while reacting the PMB or salt thereof and dextran. The subsequent introduction of sodium borohydride ($NaBH_4$) reduces the Schiff's bases and remaining aldehydes to provide stable, covalent links, preferably through one or more amine bonds, between the PMB and dextran. The conjugate is preferably purified by ultrafiltration, such as using a 10'000 molecular weight cut-off membrane, to remove residual PMB, inorganic by-products (e.g. borates), and potential low molecular weight degradation products. The pH of the aqueous medium is preferably adjusted to about 5 to 7 prior to purification.

A preferred version of the process of the present invention thus comprises:

a) preparing partially oxidized dextran by reacting dextran with sodium periodate;
b) reacting polymyxin B or a salt thereof with the resultant partially oxidized dextran in an aqueous medium at a pH of from about 9.3 to about 10 and a temperature of from about 30° to about 35°C;
c) adding sodium borohydride to the resultant aqueous medium; and
d) purifying the resultant polymyxin B - dextran conjugate.

The invention also includes water-soluble conjugates (or pharmaceutically acceptable salts thereof) of polymyxin B or a pharmaceutically acceptable salt thereof and dextran, the conjugates having anti-endotoxin activity greater than the anti-endotoxin activity of unconjugated polymyxin B, for use in the prophylactic or curative treatment of systemic inflammatory response syndrome and septic shock. It also includes such conjugates (or pharmaceutically acceptable salts thereof) obtainable by the process as defined above, and such conjugates (and pharmaceutically acceptable salts thereof) whenever obtained by the process as described above; pharmaceutical compositions comprising a water-soluble conjugate (or a pharmaceutically acceptable salt thereof) of polymyxin B or a pharmaceutically acceptable salt thereof and dextran as defined above, together with at least one pharmaceutically acceptable carrier or diluent; and the use of these conjugates (or pharmaceutically acceptable salts thereof) in the preparation of a medicament for use in the prophylactic or curative treatment of systemic inflammatory response syndrome and septic shock.

In the process of the present invention, as well as in the conjugates produced thereby, the molecular ratio of PMB or salt thereof to dextran is from about 1 : 15 to about 200 : 1; more preferably from about 1 : 2 to about 1 : 5; most preferably about 1.5 : 5.

A salt of polymyxin B preferably is a pharmaceutically acceptable salt of PMB.

A conjugate is to be viewed as being water-soluble if it has a solubility in water of about 25 mg/ml or greater at 20°C; preferably about 50 mg/ml or greater; more preferably about 60 mg/ml or greater; most preferably about 65 mg/ml or greater, at 20°C.

The PMB-dextran conjugates (and salts thereof) prepared according to the present invention can be used in a manner consistent with the use of PMB itself, namely, they can be used either alone, e.g. as an antibiotic for bacterial or fungal infections, or combined with other bactericidal and/or anti-inflammatory agents. They may be administered in any of the forms by which native PMB is conventionally administered, e.g. intramuscularly, intravenously, intrathecally, subconjunctivally or topically. Thus formulations for intramuscular injections typically comprise an effective amount of PMB - dextran conjugate (or pharmaceutically acceptable salt thereof) in sterile water, physiological saline or approximately 1% procaine.HCl. Intravenous formulations typically comprise an effective

amount of conjugate in 5% dextrose and sterile water. Intrathecal formulations typically comprise an effective amount of conjugate in physiological saline. For topical ophthalmic use, an effective amount can be mixed with water or physiological saline, and optionally glycerine, and cupric sulfate for eye drops, or it may be made into an ointment or suspension. Creams, for topical applications, especially for burned areas, typically comprise an effective amount of PMB - dextran conjugate (or pharmaceutically acceptable salt thereof) in a base of inactive ingredients such as liquid petrolatum, propylene glycol, polyoxyethylene, polyoxypropylene, and emulsifying wax.

The purified PMB-dextran conjugates (or pharmaceutically acceptable salts thereof) of the present invention are essentially non-toxic. The amount of conjugate of the present invention to be used can be determined based on the amount of native PMB which would typically be prescribed for a particular patient, taking into account such factors as the condition being treated and age and weight of the patient, and the activity of the particular conjugate used. A dosage reduction of one-half or more compared to native PMB can be realized due to the effective increased activity of the conjugate, its reduced toxicity and its increased duration of activity.

The molecular weight of the conjugates of the present invention can be determined e.g. using size exclusion high performance liquid chromatography (SEC-HPLC, TSK-GEL G4000 PWx1 eluted with 40 mM phosphate buffer at pH 4.4, detected with low angle laser light scattering) with reference to a dextran standard having a weight average molecular weight (MW) of 79'800. The conjugates of the present invention preferably have a weight average molecular weight (MW) of from about 55'000 to about 80'000; more preferably from about 60'000 to about 80'000; most preferably from about 63'000 to about 76'000.

The improved conjugates of the present invention have better reproducibility over the conjugates of the prior art and have anti-endotoxin activity greater than the anti-endotoxin activity of unconjugated polymyxin B. The conjugates of the present invention have improved anti-endotoxin activity, namely the ratio of the IC_{50} of unconjugated polymyxin B to the IC_{50} of the inventive conjugates is greater than 1.0; preferably greater than about 1.25 or 1.3; more preferably greater than about 1.4. As used herein, IC_{50} means the concentration of polymyxin B as measured by a carbocyanine dye assay which abrogates

50% of the absorbance shift of the carbocyanine dye at 450 nm in the presence of 0.05 mg of endotoxin from E. coli. As used herein, activity (%) is defined as follows:

$$\frac{IC_{50} \text{ of unconjugated polymyxin B}}{IC_{50} \text{ of polymyxin B/dextran conjugate}} \times 100$$

The carbocyanine dye assay is an in vitro biochemical assay utilized to measure the anti-endotoxin properties of polymyxin B and the conjugates of the present invention. This assay is modified from previous reports (Ogawa and Kanoh, Microbiol.Immunol. 28 [1984] 1313-1323; Zey and Jackson, Applied Microbiol. 26 [1973] 129-133) and optimized for use in microtiter plates. In this assay the conjugate or the unconjugated PMB (positive control) competes with the carbocyanine dye (a small cationic compound which changes in spectrophotometric absorbance when bound to endotoxin) for endotoxin.

Preparation of reagents for the carbocyanine dye assay is as follows:

1) A 0.15 M sodium acetate solution and a 0.15 M acetic acid solution is prepared. The two are combined (40.2 ml of 0.15 M sodium acetate and 159.8 ml of 0.15 M acetic acid) to make a 0.03 M acetate buffer;
2) 8.0 mg carbocyanine dye is dissolved in 12.5 ml of ethyl alcohol. This is diluted with 37.5 ml of acetate buffer. This is the final dye solution at 160 µg/ml in 25% ethanol. It is kept on ice, in the dark, and mixed for at least 30 minutes;
3) serial dilutions of PMB or each conjugate are prepared in 40 mM phosphate buffer (pH=7.4). Each dilution is 6-fold greater than its final testing concentration;
4) after sonication, E. coli 0111:B_4 endotoxin is diluted to 0.5 mg/ml 0.9% saline from a previously prepared, frozen stock concentration of 1.0 mg/ml 0.9% saline.

The plates are set up and the assay is performed as follows, with the addition of samples or buffer to microtiter wells first:

a) blank: 100 µl of saline and 50 µl of 40 mM phosphate buffer (pH=7.4) in triplicate;
b) positive control: 100 µl of endotoxin and 50 µl of 40mM phosphate buffer (pH=7.4) in triplicate;
c) PMB samples: 100 µl of endotoxin and 50 µl of PMB samples, in triplicate;
d) negative controls: 100 µl of saline and 50 µl of conjugate samples, in triplicate;
e) experimental: 100 µl of endotoxin and 50 µl of conjugate samples, in triplicate.

The plates are rotated for several minutes. 150 µl of dye is added to each well and the plate wrapped in foil and kept on ice and in the dark for 60 minutes. Wells are mixed with a multichannel pipette and read immediately at 450 nm on a microtiter plate reader. Reed-Muench calculations for IC_{50} estimation are performed.

The invention is illustrated by the following non-limiting Examples. All temperatures are in degrees Centigrade.

Example 1

A) Oxidation of dextran

A 5-l, 4-necked round-bottomed flask is charged with 2.31 l of water for injection and 150 g dextran. The mixture is stirred at 21°-24° at approximately 140 rpm to effect dissolution. A solution of 3.9 g sodium periodate in 60 ml of water for injection is added (mildly exortherm, approximately 1°). The addition funnel is rinsed with 30 ml of water for injection. The resulting mixture is stored at 21°-24° for 1 hour. The solution is vacuum-filtered through a 0.22 µ Corning cellulose acetate 90 mm membrane filter and warmed to 30°-32° and maintained at that temperature.

B) Preparation of polymyxin B sulfate / borate buffer solution

This preparation is started 45 min prior to the completion of the dextran oxidation, filtration and warming from A) above. A 12-l, 4-necked round-bottomed flask is charged with 3.00 l of pH 9.7 borate buffer, and the temperature of the buffer is adjusted to 32°. Next, 45 g polymyxin B sulfate is added, and the resulting suspension is stirred at 32° for 45 min. The pH of the mixture is measured (initially 9.4) and adjusted to pH 9.7 at 32° by adding 30 ml of 5 N NaOH.

The borate buffer is prepared as follows: a heat-treated 5-l, 4-necked round-bottomed flask is charged with 114.3 g of sodium tetraborate decahydrate and 2.906 l (2.90 kg) of water for injection. The solution is heated to 32°C and the pH adjusted to 9.7 (initially 9.3) by adding 37 ml of 5 N NaOH. The solution is vacuum-filtered through a sterile 0.22 µ Corning cellulose acetate 90 mm membrane filter and stored.

C) Reaction of polymyxin B sulfate with oxidized dextran

The pre-warmed (30°-32°), oxidized dextran solution (from part A) is added as rapidly as possible to the stirred mixture of polymyxin B sulfate in borate buffer at 32° (from part B). After the addition is complete, the pH of the mixture is measured (initially 9.5) and adjusted to pH 9.7 by adding 18 ml of 5 N NaOH. The mixture is stirred at 32° for 1 hour. To the heterogeneous mixture is added a solution of 3.6 g sodium borohydride in 50 ml of water for injection. The mixture is stirred at 32° for 2 hours. To the mixture is added another freshly prepared solution of 3.6 g of sodium borohydride in 50 ml of water for injection. The mixture is stirred for 2 hours at 32°. To the mixture is added a solution of 3.6 g sodium borohydride in 50 ml of water. The heating is removed and the reaction mixture is allowed to cool to ambient temperature with stirring over 14 hours. The pH of the reaction mixture is measured (10.0) and adjusted to pH 5.7 by the addition of 1.27 l of 1 N HCl. The resulting mixture is stirred for 10 min after acidification, and vacuum-filtered through a sterile 0.22 µ Corning cellulose acetate membrane filter to produce 6.81 l of a solution of polymyxin B - dextran conjugate which is held for purification.

D) Purification by ultrafiltration

The solution of polymyxin B - dextran conjugate (from part C) is purified using a pre-treated Amicon CH2PRS Ultrafiltration Unit with S1Y10 membrane cartridges. The purified product is removed from the ultrafiltration unit and stored frozen at -25°.

Analysis of the conjugate obtained showed that the product has the following characteristics:

IC_{50}= 16.8 µg/ml
activity = 124.7 %
dextran content = 65.37 mg/ml
total polymyxin B content = 2.47 mg/ml
free polymyxin B content = 0.65 %
ratio PMB: dextran = 37.71 mg PMB / g dextran.

It appears that at a reaction temperature of 32° and pH 9.5 the product obtained has an activity which is about 125 % of the activity of unconjugated PMB.

Example 2:

The process of Example 1 is repeated and yields a polymyxin B - dextran conjugate having the following characteristics:

IC_{50} = 16.4 µg/ml
activity = 125.8 %
dextran content = 65.30 mg/ml
total polymyxin B content = 2.49 mg/ml
free polymyxin B content = 0.67 %
ratio PMB : dextran = 38.06 mg PMB/g dextran

Again, at a reaction temperature of 32° and pH 9.5 the conjugate obtained has about 126 % activity as compared to unconjugated PMB.

Example 3:

Polymyxin B / dextran conjugates are prepared according to the process of Example 1 (except as indicated) to determine the effects of the temperature of the reaction between PMB and dextran at pH 9.7. Payload (mg PMB/g dextran), average IC_{50} (µg/ml) and activity (%) are determined and the results are presented in Table 1:

Table 1: Effect of temperature at pH 9.7

(10 g PMB; 0.65 g $NaIO_4$; 50 g dextran)

Example No.	Temperature	Payload (mg PMB/gdextran)	Average IC_{50} (µg/ml)	Activity (%)
3a	35°	15.87	25.18	99.7
3b	35°	19.81	26.07	91.1
3c	35°	18.58	27.67	85.0
3d	10°	49.53	143.83	23.6
3e	10°	54.95	187.87	9.5
3f	10°	56.03	195.7	10.8

From Example 3 it is apparent that when the reaction temperature is lowered to 10° at a pH of 9.7, the product obtained has reduced activity.

Example 4:

Polymyxin B / dextran conjugates are prepared according to the process of Example 1 (except as indicated) to determine the effect of the pH of the reaction between PMB and dextran. Payload (mg PMB / g dextran), average IC_{50} (µg/ml) and activity (%) are determined as in Example 3, and the results are presented in Table 2:

Table 2: Effect of pH at temperature 32°

(15g PMB; 1.3 g $NaIO_4$; 50g dextran)

Example No.	pH of reaction	Payload (mg PMB / g dextran)	Average IC_{50} (µg/ml)	Activity (%)
4a	4.0[a)]	2.36	ND	ND
4b	5.0[a)]	14.34	ND	ND
4c	7.0[b)]	15.26	ND	ND
4d	7.0[b)]	16.09	164.4	11.0
4e	8.0[b)]	32.03	107.7	17.8
4f	8.0[b)]	29.51	139.4	13.8
4g	8.5[c)]	69.94	74.5	24.9
4h	9.0[c)]	50.99	22.6	95.3
4i	9.0[c)]	50.16	22.5	88.6
4j	9.3[c)]	43.33	19.4	94.0
4k	9.3[c)]	39.96	19.0	95.8
4l	9.5[c)]	38.22	17.5	100.5
4m	9.5[c)]	39.9	17.8	98.1
4n	9.7[c)]	36.74	15.8	133.2
4o	9.7[c)]	34.99	14.9	148.9
4p	10.0[c)]	31.89	18.7	97.3

a) acetate buffer; b) phosphate buffer; c) borate buffer;
ND: not determined (samples are too dilute to determine these parameters)

The data in Table 2 shows that decreasing reaction temperature to 32° while using a pH comprised between 9.5 and 9.7 still allows production of conjugate having an activity which is superior to that of unbound PMB (Examples 4l, 4n and 4o), while increasing the pH further, to pH 10, is not particularly beneficial (Example 4p).

Example 5:

Polymyxin B / dextran conjugates are prepared as in Example 1 (except as indicated) and their anti-endotoxin activity measured. The results are presented in Table 3 below:

Table 3: Effect of the amount of PMB

(1.3 g $NaIO_4$: pH 9.7; 32°)

Example No.	Conditions	Payload (mg PMB / g dextran	Average IC_{50} (µg/ml)	Activity (%)
5a	15 g PMB	36.74	15.8	133.2
5b	15 g PMB	34.99	14.9	148.9
5c	15 g PMB	33.81	17.6	109.0
5d	20 g PMB	46.06	19.7	121.5
5e	20 g PMB	44.63	19.5	115.5

The results in Table 3 further confirm that the optimum conditions reside in a reaction temperature of about 32° and a pH of about 9.5 to about 9.7.

Overall, the data in Examples 1 to 5 thus shows that using a reaction temperature from about 30° to about 35°, preferably about 32°, and a pH from about 9.3 to about 10, preferably about 9.5 to about 9.7, results in the improved conjugates of the present invention.

This Sheet Has Been Replaced

Claims:

1. A **water-soluble conjugate** (or salt thereof) comprising polymyxin B or a salt thereof and dextran, the conjugate having anti-endotoxin activity greater than the anti-endotoxin activity of unconjugated polymyxin B, namely, wherein the ratio of the IC_{50} of unconjugated polymyxin B to the IC_{50} of the conjugate is greater than 1.0.

2. The conjugate (or salt thereof) of claim 1 wherein the ratio is greater than about 1.25 or 1.3.

3. The conjugate (or salt thereof) of claim 1 wherein the ratio is greater than about 1.4.

4. The conjugate (or salt thereof) of claim 1 wherein the anti-endotoxin activity is measured by a carbocyanine dye assay wherein the IC_{50} of the conjugate is measured as the concentration of the conjugate which abrogates 50% of the absorbance shift of the carbocyanine dye at 450 nm in the presence of 0.05 mg of endotoxin from E. coli per ml of 0.9 % saline.

5. The conjugate (or salt thereof) of any one of claims 1 to 4 wherein the molecular ratio of the polymyxin B or salt thereof to dextran is from about 1 : 15 to about 200 : 1.

6. The conjugate (or salt thereof) of any one of claims 1 to 4 wherein the molecular ratio of polymyxin B or salt thereof to dextran is from about 1 : 2 to about 1 : 5.

7. The conjugate (or salt thereof) of any one of claims 1 to 4 wherein the dextran has a weight average molecular weight of from about 25'000 to about 500'000.

8. The conjugate (or salt thereof) of any one of claims 1 to 4 wherein the dextran has a weight average molecular weight of from about 50'000 to about 200'000.

9. The conjugate (or salt thereof) of any one of claims 1 to 4 wherein the dextran has a weight average molecular weight of from about 63'000 to about 76'000.

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10. The conjugate (or salt thereof) of any one of claims 1 to 4 which has a weight average molecular weight of from about 55'000 to about 80'000.

11. The conjugate (or salt thereof) of any one of claims 1 to 4 which has a weight average molecular weight of from about 60'000 to about 80'000.

12. The conjugate (or salt thereof) of any one of claims 1 to 4 which has a weight average molecular weight of from about 63'000 to about 76'000.

13. A process for producing a water-soluble conjugate (or salt thereof) of polymyxin B or a salt thereof and dextran which comprises reacting polymyxin B or a salt thereof with dextran in an aqueous medium at a pH of from about 9.3 to about 10 and a temperature of from about 30° to about 35°C.

14. The process of claim 13 wherein the pH is from about 9.5 to about 9.7.

15. The process of claim 13 wherein the temperature is about 32°C.

16. The process of claim 13 wherein reacting polymyxin B or a salt thereof with dextran comprises covalently attaching polymyxin B or a salt thereof to dextran through one or more amine bonds.

17. The process of claim 13 wherein the polymyxin B salt is polymyxin B sulfate.

18. The process of claim 13 wherein the molecular ratio of polymyxin B or salt thereof to dextran is from about 1 : 15 to about 200 : 1.

19. The process of claim 13 wherein the molecular ratio of polymyxin B or salt thereof to dextran is from about 1 : 2 to about 1 : 5.

20. The process of claim 13 wherein the molecular ratio of polymyxin B or salt thereof to dextran is about 1.5 : 5.

21. The process of claim 13 wherein dextran has a weight average molecular weight of from about 25'000 to about 500'000.

22. The process of claim 13 wherein dextran has a weight average molecular weight of from about 50'000 to about 200'000.

23. The process of claim 13 wherein dextran has a weight average molecular weight of from about 63'000 to about 76'000.

24. The process of claim 13 wherein dextran has been partially oxidized.

25. The process of claim 24 wherein the dextran has been partially oxidized using sodium periodate.

26. The process of claim 13 wherein the pH is maintained using a borate buffer.

27. The process of claim 26 wherein the borate buffer comprises sodium tetraborate.

28. The process of claim 13 comprising subsequent reaction with sodium borohydride.

29. The process of claim 13 additionally comprising subsequently adjusting the pH of the aqueous medium to about 5 to 7 and purifying the resulting conjugate.

30. The process of claim 29 wherein the conjugate is purified via ultrafiltration.

31. A process according to claim 13 for producing a water-soluble conjugate (or salt thereof) of polymyxin B or a salt thereof and dextran which comprises:

a) preparing partially oxidized dextran by reacting dextran with sodium periodate;

b) reacting polymyxin B or a salt thereof with the resultant partially oxidized dextran in an aqueous medium at a pH of from about 9.3 to about 10 and a temperature of from about 30° to about 35°C;

c) adding sodium borohydride to the resultant aqueous medium; and

d) purifying the resultant conjugate.

32. A pharmaceutical composition comprising a water-soluble conjugate (or pharmaceutically acceptable salt thereof) of polymyxin B or a pharmaceutically acceptable salt thereof and dextran, the conjugate having anti-endotoxin activity as defined in claim 1, together with at least one pharmaceutically acceptable carrier or diluent.

33. A water-soluble conjugate (or pharmaceutically acceptable salt thereof) of polymyxin B or a pharmaceutically acceptable salt thereof and dextran, the conjugate having anti-endotoxin activity as defined in claim 1, for use in the prophylactic or curative treatment of systemic inflammatory response syndrome or septic shock.

34. A water-soluble conjugate (or pharmaceutically acceptable salt thereof) of polymyxin B or a salt thereof and dextran as defined in claim 1 obtainable by the process of claim 13 or 31.

35. A water-soluble conjugate (or pharmaceutically acceptable salt thereof) of polymyxin B or a salt thereof and dextran as defined in claim 1, whenever obtained by the process of claim 13 or 31.

36. Use of a water-soluble conjugate (or pharmaceutically acceptable salt thereof) of polymyxin B or a pharmaceutically acceptable salt thereof and dextran, the conjugate (or salt thereof) having anti-endotoxin activity as defined in claim 1, in the preparation of a medicament for use in the prophylactic or curative treatment of systemic inflammatory response syndrome or septic shock.

REPLACEMENT SHEET

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Claims:

1. A **water-soluble conjugate** (or salt thereof) comprising polymyxin B or a salt thereof and dextran, the conjugate having anti-endotoxin activity greater than the anti-endotoxin activity of unconjugated polymyxin B, namely, wherein the ratio of the IC_{50} of unconjugated polymyxin B to the IC_{50} of the conjugate is greater than about 1.4, whereby the anti-endotoxin activity is measured by a carbocyanine dye assay wherein the IC_{50} of the conjugate is measured as the concentration of the conjugate which abrogates 50% of the absorbance shift of the carbocyanine dye at 450 nm in the presence of 0.05 mg of endotoxin from E. coli per ml of 0.9 % saline; the molecular ratio of polymyxin B or salt thereof to dextran is from about 1 : 2 to about 1 : 5; the dextran has a weight average molecular weight of from about 50'000 to about 200'000; and the conjugate (or salt thereof) has a weight average molecular weight of from about 60'000 to about 80'000.

2. A process for producing a water-soluble conjugate (or salt thereof) of polymyxin B or a salt thereof and dextran which comprises reacting polymyxin B or a salt thereof with dextran in an aqueous medium at a pH of from about 9.3 to about 10 and a temperature of from about 30° to about 35°C, whereby polymyxin B or a salt thereof is covalently attached to dextran through one or more amine bonds; the molecular ratio of polymyxin B or salt thereof to dextran is from about 1 : 2 to about 1 : 5; dextran has a weight average molecular weight of from about 50'000 to about 200'000; and, optionally, dextran has been partially oxidized.

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3. A process according to claim 2 for producing a water-soluble conjugate (or salt thereof) of polymyxin B or a salt thereof and dextran which comprises:

a) preparing partially oxidized dextran by reacting dextran with sodium periodate;

b) reacting polymyxin B or a salt thereof with the resultant partially oxidized dextran in an aqueous medium at a pH of from about 9.3 to about 10 and a temperature of from about 30° to about 35°C;

c) adding sodium borohydride to the resultant aqueous medium; and

d) purifying the resultant conjugate.

4. A **pharmaceutical composition** comprising a water-soluble conjugate (or pharmaceutically acceptable salt thereof) of polymyxin B or a pharmaceutically acceptable salt thereof and dextran, as defined in claim 1, together with at least one pharmaceutically acceptable carrier or diluent.

5. A water-soluble conjugate (or pharmaceutically acceptable salt thereof) of polymyxin B or a salt thereof and dextran as defined in claim 1, whenever obtained by the process of claim 2 or 3.

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