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(54) Title: AMPHOTERICIN B AQUEOUS COMPOSITION

(57) Abstract: A low toxicity parenteral dimethyl sulfoxide free aqueous compositions containing Amphotericin B are described. The compositions essentially consist of in addition to Amphotericin B, phospholipids and sodium chloride. The compositions are sterilised by autoclaving. The process of making these compositions without the use of solvents for dissolving Amphotericin B have been described. The compositions are indicated for the treatment of invasive fungal infections.

AMPHOTERICIN B AQUEOUS COMPOSITION

Field of Invention

This invention relates to low toxicity Amphotericin B aqueous composition. This invention is particularly related to the low toxicity Amphotericin B aqueous composition containing phospholipids suitable for parenteral administration.

Background of the Invention

Amphotericin B is a polyene antifungal, antibiotic drug useful in treatment of invasive fungal infections. However, it has high nephrotoxicity.

10

The toxicity of the Amphotericin B is reduced by various processes; of these (a) entrapping the drug in liposomes and (b) converting the drug into High drug lipid complex (HDLC) are commonly used.

15 **Preparation of liposomal Amphotericin B:**

In US patent 4973465 (1990) preparation of HDLCs have been described in which sterols like cholesterol are used either alone or in combination with natural phospholipids, phosphatidylcholine.

20 In US patent 5616334 (1997) a method of preparing liposomal Amphotericin B which involves initially producing blank multilamellar vesicles (MLVs) and then mixing the MLVs with sonicated Amphotericin B suspension in water has been described. This process does not involve the use of any solvents. However, this procedure specifically produces liposomal Amphotericin B which is more toxic than Amphotericin B HDLC. The 25 procedure involves extrusion of blank liposomes for sizing through stacked polycarbonate filters again and again ten times. It also involves removal of unincorporated Amphotericin B by centrifugation after drug loading.

30 This US patent also describes a process for making HDLC in "Low toxicity drug-lipid systems". In this patent a process for the preparation of Amphotericin B lipid complex has been described. This technique in general is as follows:

Preparation of HDLCs : First the drug Amphotericin B is solubilised in a solvent such as Dimethyl sulfoxide (DMSO) or methanol. The lipids, preferably dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) in a molar ratio of 7 : 3 are solubilised in solvents such as methanol, ethanol, chlorinated hydrocarbons. The drug solution and the lipid solution are mixed. The solvents are evaporated under reduced pressure, resulting in a thin lipid-drug film. The film is hydrated with an aqueous solution such as water, saline, phosphate buffer saline or glycine buffer, to form HDLCs.

10 In one variation of the above process, the resulting dry lipid - drug film is resuspended in a solvent, such as methylene chloride and again evaporated under reduced pressure prior to hydrating the film.

15 In another variation of the above process, the dry lipid-drug film is dehydrated to form flakes; the flakes are then hydrated with aqueous solution.

In another process, the aqueous solution such as saline, buffer or water is added to the solution containing the drug and the lipid, and then the solvent is evaporated off to obtain HDLCs. In this process, formation of thin film of the phospholipids is not required.

20 In an alternative method for forming the HDLCs described in this US patent, lipid particles (or liposomes) containing bioactive agents, such as Amphotericin B, are formed by first making multilamellar vesicles (MLVs) containing from 6 - 50 mole percent of the bioactive agent. Then subjecting the MLVs to a heating cycle, from about 25°C to about 25 60°C, most preferably about 60°C. Such a cycle forms a more highly ordered and less toxic Amphotericin B lipid complex.

30 Another alternate process of making Amphotericin B lipid complex has also been described in this US patent. In that process lipids are admixed with sodium chloride solution (0.9%) and homogenised using a homogeniser. Amphotericin B is dissolved in DMSO and added to the lipid solution while homogenising and homogenised further for about thirty minutes, until the particle size is reduced to about less than 10 microns,

preferably to about 10 micron. The resulting lipid particles are size selected following tangential flow filtration. The disadvantage with this process is that the solvent used DMSO has high boiling point and hence difficult to remove from the product. Further trace quantity of DMSO remains in the final product. It is not desirable to have such a solvent in 5 trace quantities in the composition for intravenous administration, as this solvent has been reported to be hepatotoxic (The journal of Infectious diseases 1991 : 164 Pg 418 to 421).

10 HDLCs are useful preparations to reduce toxicity of Amphotericin B, but the techniques described in US patent 5616334 (1997) require use of large amount of organic solvents, as Amphotericin B has a low solubility in most of the commonly used parenterally acceptable organic solvents. Hence the process involves removal of large quantities of 15 organic solvents by evaporation. Alternatively aprotic solvents such as dimethyl sulfoxide, dimethyl formamide are also used to dissolve Amphotericin B. These aprotic solvents have high boiling point and traces of these solvents is bound to remain in the final composition. As these aprotic solvents are reported to be hepatotoxic, it is not desirable to use these 15 solvents in the process of manufacturing.

20 There is, therefore, a need to improve the process for large scale manufacture of such Amphotericin B compositions by reducing the quantity of solvents used. That will also bring down the production cost.

25 The main object of the present invention is to develop a low toxicity parenteral aqueous composition containing Amphotericin B and phospholipids with a view to make it simple and to reduce the cost of manufacture. Further extension of the main object of the present invention is to develop a low toxicity parenteral aqueous composition containing Amphotericin B and phospholipids and not containing traces of DMSO and / or chlorinated 25 hydrocarbons.

Summary of the Invention :

30 Accordingly, the present invention relates to a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids.

The present invention further relates to a process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids comprising steps of

- (i) dissolving one or more phospholipids in one or more of organic solvents selected from a group of parenterally acceptable solvents such as methanol, ethanol, isopropyl alcohol, chloroform, carbon tetrachloride and methylene chloride and then removing the solvents by evaporation under reduced pressure to form a dry film of the single or mixed phospholipids;
- (ii) suspending Amphotericin B in a parenterally acceptable aqueous phase, not containing sodium chloride or suspending micronised Amphotericin B in a parenterally acceptable aqueous phase, which may contain sodium chloride;
- (iii) adding aqueous phase containing suspended Amphotericin B formed at the end of step (ii) to said film of phospholipids obtained at the end of step (i) and mixing the two to obtain a suspension of said Amphotericin B together with said phospholipids in said aqueous phase;
- (iv) adjusting the pH of said suspension obtained at the end of step (iii) to 6.0 - 8.0 and then homogenising it till it becomes filterable through a 2μ glass fibre filter;
- (v) adding sufficient sodium chloride solution in water at the end of step (iv) so that the sodium chloride content of the final product is at least 0.1% w/v;
- (vi) filtering said homogenised suspension obtained at the end of step (v) through a 2μ glass fibre filter and filling the filtrate in vials under nitrogen cover, sealing the vials and sterilising the sealed vials by autoclaving to obtain the final product suitable for parenteral administration.

The present invention also relates to a low toxicity parenteral Amphotericin B aqueous composition, containing atleast 0.1% w/v sodium chloride and phospholipids as described herein and made by the process of the present invention as described above.

Detailed description of embodiments of the invention

The content of Amphotericin B in the composition of present invention varies from 0.1% w/v to 1.0% w/v of the composition, preferably the content of Amphotericin B is 0.5%w/v of the composition.

The total content of phospholipids varies from 0.1% w/v to 1.0%w/v of the composition. The preferred content is from about 0.4% to about 0.6%w/v.

5 The weight ratio of Amphotericin B to phospholipids is from about 1:0.5 to about 1:1.5. The preferred weight ratio is from about 1:0.8 to about 1:1.2.

10 In this process, phospholipids are chosen from egg phosphatidylcholine, or a mixture of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol sodium salt (DMPG). When a mixture of two phospholipids DMPC and DMPG are used, then the weight ratio of phospholipids DMPC : DMPG is between 7:1 and 7:15, preferably 15 7:3.

15 The solvents used for dissolving the phospholipids are chosen from alcoholic solvents such as ethanol, methanol, Isopropyl alcohol with or without addition of chlorinated hydrocarbons such as chloroform, methylene chloride, carbon tetrachloride. Alcoholic solvents alone or chlorinated hydrocarbons alone can be used for dissolving the phospholipids. Alternatively alcoholic solvents and chlorinated hydrocarbons can also be used in combination to dissolve the phospholipids. When chlorinated hydrocarbons are not selected, the composition is free from chlorinated hydrocarbons. Preferred solvent used for 20 dissolving phospholipids is ethanol.

Micronised Amphotericin B wherever used in this invention is micronised using air jet mill to particle size less than 10 microns.

25 The pH of aqueous phase used for dispersing Amphotericin B is adjusted to 6.0 - 8.0 using dilute sodium hydroxide solution whenever buffer solution is not used in the composition.

30 The aqueous phase used for suspending Amphotericin B is parenterally acceptable vehicle such as water or phosphate buffer. When micronised Amphotericin B is used, the aqueous phase used for suspending Amphotericin B can be saline, phosphate buffer saline, water or phosphate buffer.

Sodium chloride is added as a solution in water after homogenisation and before filtration. However sodium chloride can be added at any step (ii) to (iv) of manufacturing specified under "Summary of the invention" when micronised Amphotericin B is used.

5 The concentration of Sodium chloride is from about 0.1% to 0.9% w/v of the composition preferably 0.4% to 0.9% w/v of the composition.

Homogenisation is carried out using high pressure homogeniser at not less than 5000 psi till the product is filterable through 2 micron glass fibre filter.

10

In another embodiment of the invention, the Amphotericin B lipid suspension is sonicated in a bath sonicator before homogenisation to get the uniform suspension after adjusting the pH to about 6.0 - 8.0. Dilute sodium hydroxide solution is used to adjust the pH whenever buffer solution is not used in the composition.

15

The homogenised Amphotericin B lipid suspension is filtered through 2μ glass fibre filters following the usual filtration procedure under pressure either using filtered Nitrogen or filtered compressed air.

20

After filtration, the homogenised suspension is filled into vials under nitrogen cover and sterilised by conventional autoclaving at 110°C to 121°C, preferably at 121°C for 20 minutes or 110°C for 40 minutes. The sterilisation can also be carried out by specialised process of autoclaving in which the heating and cooling cycle time is reduced by rapid heating and rapid cooling system.

25

In the earlier process of preparing HDLCs as described in US patents 4973465 (1990) and 5616334 (1997), Amphotericin B is dissolved in very large amount of organic solvents. In one of the examples in US patent 5616334 (1997), for preparing 1 vial of 20 ml of Amphotericin B lipid complex equivalent to 100mg of Amphotericin B, one litre of 30 methanol is used. In another example to decrease the solvent volume, 5 ml of DMSO for 100mg of Amphotericin B has been used, but DMSO is not a recommended solvent for intravenous injection as DMSO has been reported to be hepatotoxic.

In the process of the present invention, Amphotericin B is not at all dissolved in any solvents while the conventional process use DMSO for dissolving Amphotericin B.

5 In the process of present invention, when Amphotericin B, suspended in an aqueous phase containing sodium chloride, was added to the lipid film and homogenised, it was observed to form aggregates and the homogenised product was not filterable through 2 micron glass fibre filter by the usual filtration procedure.

10 After extensive experimentation, we found that when aqueous phase used for suspending Amphotericin B was prepared without addition of any sodium chloride in it, homogenisation proceeded smoothly without any aggregation of the suspension and the homogenised bulk was filterable.

15 However, during the course of this invention, we found that sodium chloride is essential in the composition to reduce toxicity. Amphotericin B aqueous compositions containing different concentrations of sodium chloride at a dose of 80 mg/kg body weight, were injected in mice, in a group of eight. Amphotericin B aqueous composition without any sodium chloride as described in the Examples below were prepared and injected separately. Before each injection, volume equivalent to a dose of 80 mg/kg body weight 20 was diluted to 0.5 ml with 5% dextrose injection to render it isotonic. The percentage mortality observed at the end of 72 hours are as shown in Table 1.

Table 1

Concentration of Sodium chloride in Amphotericin B aqueous composition	Prepared as per Example	Percentage mortality in mice at 80mg/kg dose
0.9% w/v	III	Nil
0.7% w/v	IV	Nil
30 0.4% w/v	V	Nil
0.1% w/v	VI	50%
Nil	XIII	87.5%

From the Table 1, it is clear that a minimum concentration of 0.1% sodium chloride is essential to reduce toxicity. Hence during the hydration of phospholipid film, aqueous phase without any sodium chloride was used to make homogenisation smooth and filtration through 2μ glass fibre filter easy. Sodium chloride was added as a solution after the 5 process of homogenisation. Addition of Sodium chloride in the composition of the present invention is essential to reduce toxicity of Amphotericin B. Eventhough with the addition of 0.1% w/v sodium chloride, the LD₅₀ was 80mg/kg, this LD₅₀ is very much higher than the conventional Amphotericin B preparations containing sodium desoxycholate which is reported to be around 4mg/kg.

10

After a lot of further experiments, we found that reducing the average particle size of Amphotericin B to less than 10 microns by micronising, helped in overcoming the problem of aggregation during homogenisation. The particle size analysis of Amphotericin B before and after micronisation was carried out with Sympatic HELOS Particle Size Analyser. 15 Micronisation of Amphotericin B also helped in overcoming the problem of filtration associated with the presence of sodium chloride in the homogenised aqueous suspension containing non-micronised Amphotericin B and phospholipid.

20 Filtration of homogenised bulk prepared using micronised Amphotericin B is easy and commonly used filters can be used; in the prior art process such as in US patent 5616334 (1997), filtering of HDLC is performed through a torturous path or straight through a membrane filter such as a polycarbonate filter.

25 Thus in one embodiment of the invention, we could overcome the problem of aggregation and filtration using non-micronised Amphotericin B by avoiding addition of sodium chloride to the aqueous phase. However, we found that addition of sodium chloride in atleast a minimum concentration of 0.1% w/v is essential to reduce the toxicity of Amphotericin B aqueous composition. Having sodium chloride in it, we solved the problem of aggregation and filtration by deferring the addition of Sodium chloride until the 30 step of homogenisation.

Accordingly, in the first embodiment of the invention, in the process for manufacture of a low toxicity sterile Amphotericin B aqueous composition containing

atleast 0.1% w/v sodium chloride, the aqueous phase used for dispersing non-micronised Amphotericin B is water or phosphate buffer not containing sodium chloride. Sodium chloride is added just after homogenisation of the aqueous suspension containing non-micronised Amphotericin B and the phospholipids.

5

In the second embodiment of the invention, the problem of aggregation and filtration of the suspension of the phospholipid and the aqueous phase containing Amphotericin B, was solved by using micronised Amphotericin B.

10

In combination of the two embodiments, when the Amphotericin B used is micronised and is suspended in an aqueous phase not containing sodium chloride, sodium chloride is added into the aqueous phase before, during or after homogenisation step.

Examples :

15

The invention will now be illustrated by way of Examples. The Examples are by way of illustration only and in no way restrict the scope of the invention.

There are 4 groups of Examples as described below in the Table 2.

Table 2

20

Group	Example Nos.	Amphotericin B	Aqueous phase used for dispersing Amphotericin B	Addition of salt	Embodiment of the invention
25	A	I - II	Non-micronised	Not containing salt	After homogenisation step
	B	III - VI } VIII - IX }	Micronised	Containing salt	-----
30		VII*	Micronised	Containing salt	-----
	C	X - XII	Micronised	Not containing salt	During / after Homogenisation step
35	D	XIII	Micronised	-----	First & second
		XIV	Non-micronised	Not containing salt	-----
40	*	Solvent used for dissolving phospholipids is only ethanol.			

All the raw materials used in these Examples were of parenteral grade. Equipments used were of conventional nature. Entire processing was done in an area with a controlled environment required for manufacturing sterile products.

5

Amphotericin B used in these Examples was of parenteral grade obtained from Alpharma complying with USP specifications. Micronised Amphotericin B wherever used in these Examples was prepared by micronising Amphotericin B using air jet mill to the particle size of less than 10 microns.

10

Phospholipids DMPC and DMPG used in the Examples were of parenteral grade and were procured from Avanti Polar Lipids.

15

Phospholipid Egg phosphatidylcholine used in the Examples was of parenteral grade and was procured from Lipoids

Organic solvents used in the Examples were of AR (Analytical reagent) quality.

20

Phosphate buffer used in the Examples were prepared as per Indian Pharmacopoeia.

Phosphate buffer saline pH 7.4 used in the Example was prepared by dissolving 1.19gm of Disodium hydrogen orthophosphate, 0.095gms of Potassium dihydrogen orthophosphate and 4gms of Sodium chloride in 400ml of water. Water was added to make up the volume to 500ml.

GROUP A : Example I & II

The ingredients used in these Examples are shown in Table 3 :

5

Table 3

			Example I	Example II
	a)	Amphotericin B	1.00g	1.00g
10	b)	DMPC	0.68g	0.68g
	c)	DMPG	0.30g	0.30g
	d)	Ethanol*	200ml	200ml
	e)	Chloroform*	10 ml	10ml
	f)	pH - at dispersion	6.95**	7.2
15		before homogenisation	6.80**	7.2
	g)	Sodium chloride	1.80g	1.80g
	h)	Water q.s.to	200ml	--
	i)	Phosphate buffer pH 7.2	q.s.to	200ml

20 * Does not remain in the final product.

** Adjusted using 0.1N Sodium hydroxide solution

Procedure :

In Example I, Amphotericin B was suspended in 150ml of water under stirring and 25 under nitrogen bubbling. The pH was adjusted to about 6.95 with 0.1 N Sodium hydroxide solution.

In Example II, Amphotericin B was suspended in 150ml of Phosphate buffer pH 7.2 under stirring and under nitrogen bubbling.

Phospholipids DMPC and DMPG were dissolved in Chloroform in a rotary flask. Ethanol was added after complete dissolution of phospholipids and allowed to mix by rotating the flask at moderate speed under nitrogen flushing. This alcoholic solution was rotary evaporated under reduced pressure to complete dryness. Nitrogen was flushed for 30 min. after complete removal of solvents.

The dry lipid film was hydrated in the rotary flask with aqueous suspension of Amphotericin B prepared as above keeping the flask under continuous rotation with continuous flushing of nitrogen. pH of Amphotericin B lipid suspension obtained in Example I was adjusted to about 6.80 with 0.1N Sodium hydroxide solution. The content of the flask was sonicated in a bath sonicator for 1 hr. The volume was made upto 180 ml with water in Example I and with phosphate buffer pH 7.2 in Example II.

The Amphotericin B Lipid suspension was then homogenised using APV high pressure homogeniser till the homogenised product was filterable through 2 μ glass fibre filter.

Sodium Chloride was dissolved in water and diluted to 20ml with water in Example I. In Example II, sodium chloride was dissolved in and diluted to 20ml with phosphate buffer pH 7.2. This sodium chloride solution was added to the homogenised Amphotericin B Lipid suspension under low speed stirring and nitrogen flushing. This product was transferred back to the homogeniser and recirculated for 5 minutes without applying pressure. Then it was filtered through a 2 μ glass fibre filter and filled into glass containers under nitrogen, sealed and autoclaved at 110°C for 40 mins. In Example II autoclaving is done at 110°C for 40 minutes with rapid heat and rapid cooling cycle.

GROUP B :1) **Example III to VI**

5 The ingredients used in these Examples are shown in Table 4 with the procedure given below, quantity of sodium chloride added has been changed from 1.8g to 0.2g.

Table 4

		Examples			
		III	IV	V	VI
10	a)	Amphotericin B (micronised)	1g	1g	1g
15	b)	DMPC	0.68g	0.68g	0.68g
	c)	DMPG	0.30g	0.30g	0.30g
	d)	Sodium Chloride	1.80g	1.40g	0.80g
	e)	Ethanol*	200ml	200ml	200ml
	f)	Chloroform*	10ml	10ml	10ml
20	g)	pH - at dispersion before homogenisation	7.20** 7.00**	7.15** 7.10**	7.05** 7.15**
	h)	Water q.s.to	200ml	200ml	200ml

* Does not remain in the final product.

25 ** Adjusted using 0.1N Sodium hydroxide solution

Procedure :

Phospholipids DMPC and DMPG were dissolved in Chloroform in a rotary flask. Ethanol was added after complete dissolution of phospholipids and allowed to mix by 30 rotating the flask at moderate speed under nitrogen flushing. This alcoholic solution was rotary evaporated under reduced pressure to complete dryness. Nitrogen was flushed for 30 min. after complete removal of solvents.

Sodium chloride was dissolved in 175ml of water. Nitrogen was bubbled in this solution for 15 min. Micronised Amphotericin B was then suspended in the sodium chloride solution under stirring and under nitrogen bubbling. The pH was adjusted by addition of 0.1N sodium hydroxide to the values as shown in Table 4 for each Example.

5

The dry lipid film was hydrated in the rotary flask with aqueous suspension of Amphotericin B prepared as above, keeping the flask under continuous rotation with continuous flushing of nitrogen. pH of this Amphotericin B lipid complex obtained was adjusted as shown Table 4. The content of the flask was sonicated in a bath sonicator for 10 hr.

10

The volume was made upto 200 ml with water.

The Amphotericin B Lipid suspension was then homogenised using high pressure 15 homogeniser till the product was filterable through 2 μ glass fibre filter.

The homogenised Amphotericin B Lipid suspension was filtered through a 2 μ glass fibre filter and filled into glass containers under nitrogen, sealed and autoclaved at 110°C for 40 mins with rapid heat and rapid cooling cycle.

20

Toxicity studies in mice with the product of Examples III, IV and V at a dose of 80mg/kg body weight did not show any mortality while that of Example VI showed 50% mortality.

Group B continued.....**2) Example VII to IX**

5 The ingredients used in these Examples are shown in Table 5 with the procedure given below

Table 5

		Examples		
		VII	VIII	IX
10	a) Amphotericin B (micronised)	1g	1g	1g
	b) DMPC	0.68g	--	0.68g
	c) DMPG	0.30g	--	0.30g
15	d) Egg phosphatidylcholine	--	0.90g	--
	e) Sodium Chloride	1.80g	1.80g	***
	f) Ethanol*	300ml	200ml	200ml
	g) Chloroform*	--	15ml	10ml
	h) pH - at dispersion	7.15**	7.15**	7.40
20	before homogenisation	7.05**	6.95**	7.40
	i) Water q.s.to	200ml	200ml	--
	j) Phosphate buffer saline q.s.to	--	--	200ml

* Does not remain in the final product.

25 ** Adjusted using 0.1N sodium hydroxide solution.

*** ≈ 2gm contributed from PBS.

Procedure :

30 In the Example VII, phospholipids DMPC and DMPG were dissolved in ethanol in a rotary flask by rotating the flask at moderate speed under nitrogen flushing.

In the Example VIII, phospholipid Egg phosphatidylcholine was dissolved in Chloroform in a rotary flask and in the Example IX phospholipids DMPC & DMPG were

dissolved in chloroform in a rotary flask. Ethanol was added after complete dissolution of phospholipids in chloroform and allowed to mix by rotating the flask at moderate speed under nitrogen flushing.

5 In these Examples the phospholipid solutions thus obtained were rotary evaporated under reduced pressure to complete dryness. Nitrogen was flushed for 30 min. after complete removal of the solvent.

10 In Example VII & VIII, sodium chloride was dissolved in 175ml of water. Nitrogen was bubbled in this solution for 15 min. Micronised Amphotericin B was then suspended in the sodium chloride solution under stirring and under nitrogen bubbling, the pH was adjusted to about 7.15 with 0.1 N Sodium hydroxide solution.

15 In Example IX, micronised Amphotericin B was suspended in 175ml of phosphate buffer saline pH 7.4 (PBS) under stirring. Nitrogen was bubbled for 15 minutes. PBS contributes about 2gms of sodium chloride.

20 The dry lipid film was hydrated in the rotary flask with aqueous suspension of micronised Amphotericin B prepared as above keeping the flask under continuous rotation with continuous flushing of nitrogen. pH of this Amphotericin B lipid suspension obtained was adjusted to 7.05 in Example VII and to 6.95 in Example VIII with 0.1N Sodium hydroxide solution.

25 The volume was made upto 200 ml with water in Example VII & VIII.
The volume was made upto 200 ml with phosphate buffer saline pH 7.4 in Example IX.

30 The Amphotericin B Lipid suspension was then homogenised using APV high pressure homogeniser till the homogenised product was filterable through 2 μ glass fibre filter.

The homogenised Amphotericin B Lipid suspension was filtered through a 2μ glass fibre filter and filled into glass containers under nitrogen, sealed and autoclaved at 121°C for 20 minutes in Example IX and at 110°C for 40 minutes in Example VII & VIII.

5 The Sterile Amphotericin B aqueous composition obtained in Example VII was subjected to toxicity studies in mice and stability studies. The results of the toxicity study are given in Table 6 and stability study are given in Table 7.

Particle size analysis :

10 Particle size of the Amphotericin B aqueous composition obtained in Example VII was evaluated on Model 770 AccuSizer of Particle Sizing Systems, Inc., USA. 95% of the particles were found to be below 1.63μ in size and 90% of the particles were found to be below 1.28μ in size.

15 *Toxicity study in mice :*

The toxicity study of the Amphotericin B aqueous composition obtained in Example VII was studied in mice along with a conventional Amphotericin B product containing sodium desoxycholate. The followings are the observations:

20

Table 6
Acute toxicity study in mice

25

LD ₅₀ (Intravenous)	-	
Amphotericin B conventional product	-	3.5mg/kg body weight
Amphotericin B aqueous composition of Example VII	-	>80mg/kg body weight

30

The LD₅₀ of Amphotericin B aqueous composition prepared in this laboratory after single injection was >80 mg/kg in mice. This was more than 20 times higher than LD₅₀ after a single injection of conventional Amphotericin B product containing sodium desoxycholate.

Table 7

**Stability data for Amphotericin B aqueous composition
of Example VII at recommended storage temperature of 2°C - 8°C**

5

	PERIOD	APPEARANCE	AMPHOTERICIN B CONTENT
10	Initial	Yellow coloured suspension which settles on keeping and disperses uniformly on mild shaking	100.6%
15	6 Months	Yellow coloured suspension which settles on keeping and disperses uniformly on mild shaking	100.3%
20	1 Year	Yellow coloured suspension which settles on keeping and disperses uniformly on mild shaking	99.8%
25	18 Months	Yellow coloured suspension which settles on keeping and disperses uniformly on mild shaking	98.3%
	2 Years	Yellow coloured suspension which settles on keeping and disperses uniformly on mild shaking	96.5%

This example clearly shows that parenteral Amphotericin B aqueous composition having not even a trace of DMSO or chlorinated hydrocarbon when prepared by the improved process of the present invention where these solvents are not at all used, complies with general requirements of a marketable injectable suspension product. The novel aqueous composition prepared by the process of Example VII is totally free from harmful solvents such as DMSO and chlorinated hydrocarbons.

GROUP C : Example X to XII

The ingredients used in these Examples are shown in Table 8 with the procedure given below

5

Table 8

		Examples		
		X	XI	XII
10	a) Amphotericin B (micronised)	1g	1g	1g
	b) DMPC	0.68g	0.68g	0.68g
	c) DMPG	0.30g	0.30g	0.30g
15	d) Sodium Chloride	1.80g	1.80g	1.80g
	e) Ethanol*	200ml	200ml	200ml
	f) Chloroform*	10ml	10ml	10ml
	g) pH - at dispersion before homogenisation	7.15** 6.90**	7.30** 7.00**	7.2 7.2
20	h) Water q.s.to	200ml	200ml	--
	i) Phosphate buffer pH 7.2 q.s. to	--	--	200ml

* Does not remain in the final product

** Adjusted using 0.1N Sodium hydroxide solution.

25

Procedure :

Phospholipids DMPC and DMPG were dissolved in Chloroform in a rotary flask. Ethanol was added after complete dissolution of phospholipids and allowed to mix by rotating the flask at moderate speed under nitrogen flushing. This alcoholic solution was rotary evaporated under reduced pressure to complete dryness. Nitrogen was flushed for 30 min. after complete removal of solvents.

Micronised Amphotericin B was suspended in 150ml of water in Example X and XI and in 150ml of phosphate buffer pH 7.2 in Example XII under stirring and under nitrogen bubbling. The pH was adjusted by addition of 0.1N sodium hydroxide to the values as shown in Table 8 for examples X & XI.

5

The dry lipid film was hydrated in the rotary flask with micronised Amphotericin B suspension prepared as above using the rotary evaporator with continuous flushing of nitrogen. pH of Amphotericin B lipid suspension obtained was adjusted with 0.1N Sodium hydroxide solution as shown in Table 8. In Examples X and XI, the volume was made upto 10 180ml with water while in Example XII, the volume was made upto 180ml with Phosphate buffer pH 7.2. In Example XI, the content of the flask was sonicated in a bath sonicator for 1 hour.

15 In Example X & XII, the Amphotericin B Lipid suspension was then homogenised using APV high pressure homogeniser till the homogenised product was filterable through 2 μ glass fibre filter.

20 Sodium chloride was dissolved in water and diluted to 20ml with water in Example X. Sodium chloride was dissolved in phosphate buffer of pH 7.2 and diluted to 20ml with phosphate buffer of pH 7.2 in Example XII. This sodium chloride solution was added to the homogenised Lipid suspension under low speed stirring and nitrogen flushing. This product was transferred back to the homogeniser and recirculated for 5 minutes without applying pressure.

25 In Example XI, the Amphotericin B lipid suspension after sonication was passed through homogeniser 3 times under pressure using APV high pressure homogeniser. Sodium chloride was dissolved in water and diluted to 20ml with water. This sodium chloride solution was added under low speed mixing to the Amphotericin B lipid suspension obtained at the end of 3 passes. This product was transferred back to the 30 homogeniser and recirculated for 5 minutes without applying pressure. This was again homogenised under pressure till the product was filterable through 2 μ glass fibre filter.

The product was then filtered through a 2 μ glass fibre filter. The filtered product was filled into glass containers under nitrogen, sealed and autoclaved at 110°C for 40 minutes with rapid heat and rapid cooling cycle.

5 **GROUP D : Example XIII and XIV**

The ingredients used in these Examples are shown in Table 9 with the procedure given below

10

Table 9

		Example XIII	Example XIV
	a) Amphotericin B (micronised)	1g	--
15	b) Amphotericin B (non-micronised)	--	1g
	c) DMPC	0.68g	0.68g
	d) DMPG	0.30g	0.30g
	e) Ethanol*	200ml	200ml
	f) Chloroform*	10 ml	10ml
20	g) Water q.s.to	200ml	200ml
	h) pH - at dispersion	7.25**	7.20**
	before homogenisation	7.15**	7.10**

* Does not remain in the final product

25 ** Adjusted using 0.1N Sodium hydroxide solution

Procedure :

Phospholipids DMPC and DMPG were dissolved in Chloroform in a rotary flask. Ethanol was added after complete dissolution of phospholipids and allowed to mix by 30 rotating the flask at moderate speed under nitrogen flushing. This alcoholic solution was rotary evaporated under reduced pressure to complete dryness. Nitrogen was flushed for 30 min. after complete removal of solvents.

In Example XIII, micronised Amphotericin B was suspended in water under stirring and under nitrogen bubbling. In Example XIV, non-micronised Amphotericin B was suspended in water under stirring and under nitrogen bubbling. The pH was adjusted with 0.1 N Sodium hydroxide solution as shown in Table 9.

5

The dry lipid film was hydrated in the rotary flask with Amphotericin B suspension prepared as above using the rotary evaporator with continuous flushing of nitrogen. pH was adjusted with 0.1N Sodium hydroxide solution as shown Table 9. In Example XIV, the content of the flask was sonicated for 1 hr. The volume was made upto 200 ml with water.

10

The Amphotericin B Lipid suspension was then homogenised using APV high pressure homogeniser till the homogenised product was filterable through 2 μ glass fibre filter.

15

The homogenised Amphotericin B Lipid suspension was filtered through a 2 μ glass fibre filter. The filtered product was filled into glass containers under nitrogen, sealed and autoclaved. Autoclaving was done at 121°C for 20 minutes.

Toxicity study in mice :

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The toxicity of Amphotericin B aqueous composition (without sodium chloride) obtained in Example XIII and XIV was studied in mice along with Amphotericin B aqueous composition containing sodium chloride as per Example III. The followings are the observations:

Table 10

25

Acute toxicity study in mice

LD ₅₀ (Intravenous)		
Amphotericin B aqueous composition (with sodium chloride) as per Example III	-	> 80mg/kg body weight
Amphotericin B aqueous composition (without sodium chloride) as per Example XIII & XIV	-	40mg/kg body weight

The LD₅₀ of Amphotericin B aqueous composition prepared without sodium chloride as per Example XIII and XIV after single injection was 40 mg/kg body weight in mice as compared to >80 mg/kg with Amphotericin B aqueous composition prepared with sodium chloride as per Example III. This proves that sodium chloride is required to form 5 Amphotericin B aqueous composition of low toxicity.

ADVANTAGES OF THE INVENTION :

The advantages of the present invention are given below :

10

i) In present invention, Amphotericin B aqueous composition has been prepared without the use of DMSO which has been reported to be hepatotoxic.

15

ii) Due to low solubility of Amphotericin B in parenterally acceptable organic solvents, (0.1 mg/ml in methanol) a large volume of organic solvent is required which makes the process more tedious, time consuming and commercially not feasible. The process of the present invention does not require, dissolving Amphotericin B in any organic solvent

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iii) The process of the prior art requires a specialised technique for filtration like tangential flow filtration or extrusion. In the process of present invention, conventional filtration is used.

25

iv) The product prepared by the process of present invention, is stable to sterilisation by autoclaving thus making it suitable for intravenous use.

30

Process of present invention is simple and cost effective, and an improvement over the process of prior art. The most important feature of the process of this invention is the greatest purity of the obtained product without the risk of retaining any traces of harmful solvents because such solvents are not at all used in the process.

CLAIMS

1. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids.

5

2. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in claim (1) wherein the phospholipids used are chosen from egg phosphatidylcholine (EPC) or a mixture of dimyristoyl phosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol sodium salt (DMPG).

10

3. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (2) wherein the content of Amphotericin B is from about 0.1% to 1% w/v of the composition.

15

4. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (3) wherein the content of Amphotericin B is 0.5% w/v of the composition.

20

5. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (4) wherein the content of Sodium chloride is atleast 0.1% w/v of the composition.

25

6. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (5) wherein the content of Sodium chloride is between 0.1% to 0.9% w/v of the composition.

30

7. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (6) wherein the content of phospholipids is from about 0.1% to 1% w/v of the composition.

8. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (7) wherein the content of phospholipids is between 0.4% to 0.6% w/v of the composition.

5

9. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (8) wherein the weight ratio of Amphotericin B to phospholipids is from about 1 : 0.8 to about 1 : 1.2.

10

10. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (9) wherein the weight ratio of phospholipids dimyristoylphosphatidylcholine (DMPC) : dimyristoylphosphatidylglycerol (DMPG) is from about 7:1 to about 15

7:15.

15

11. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (10) wherein the weight ratio of phospholipids dimyristoylphosphatidylcholine (DMPC) : dimyristoylphosphatidylglycerol (DMPG) used is 7:3.

20

12. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (11) wherein the composition is totally free from any chlorinated 25 hydrocarbon.

25

13. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (12), comprising steps of

(i) dissolving one or more phospholipids in one or more of organic solvents selected from a group of parenterally acceptable solvents such as methanol, ethanol, isopropyl alcohol, chloroform, carbon tetrachloride and

methylene chloride and then removing the solvents by evaporation under reduced pressure to form a dry film of the single or mixed phospholipids;

- 5 (ii) suspending Amphotericin B in a parenterally acceptable aqueous phase, not containing sodium chloride or suspending micronised Amphotericin B in a parenterally acceptable aqueous phase, which may contain sodium chloride;
- (iii) adding aqueous phase containing suspended Amphotericin B formed at the end of step (ii) to said film of phospholipids obtained at the end of step (i) and mixing the two to obtain a suspension of said Amphotericin B together with said phospholipids in said aqueous phase;
- 10 (iv) adjusting the pH of said suspension obtained at the end of step (iii) to 6.0 - 8.0 and then homogenising it till it becomes filterable through a 2μ glass fibre filter;
- (v) adding sufficient sodium chloride solution in water at the end of step (iv) so that the sodium chloride content of the final product is at least 0.1% w/v;
- 15 (vi) filtering said homogenised suspension obtained at the end of step (v) through a 2μ glass fibre filter and filling the filtrate in vials under nitrogen cover, sealing the vials and sterilising the sealed vials by autoclaving to obtain the final product suitable for parenteral administration.

20 14. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in claim (13) wherein the phospholipids are chosen from egg phosphatidylcholine (EPC) or a mixture of dimyristoyl phosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol sodium salt (DMPG).

25

15. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) & (14) wherein the content of Amphotericin B is from about 0.1% to 1% w/v of the composition

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16. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and

phospholipids as claimed in any of claims (13) - (15) wherein the content of Amphotericin B is 0.5% w/v of the composition.

17. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (16) wherein the content of phospholipids is from about 0.1% to 1% w/v of the composition.

18. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (17) wherein the content of phospholipids is between 0.4% to 0.6% w/v of the composition.

19. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (18) wherein the weight ratio of Amphotericin B to phospholipids is from about 1 : 0.8 to about 1 : 1.2.

20. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (19) wherein the weight ratio of phospholipids dimyristoylphosphatidylcholine (DMPC) dimyristoylphosphatidylglycerol (DMPG) is from about 7:1 to about 7:15.

25 21. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (20) wherein the weight ratio of phospholipids dimyristoylphosphatidylcholine (DMPC) dimyristoylphosphatidylglycerol (DMPG) used is 7:3.

30 22. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and

phospholipids as claimed in any of claims (13) - (21) wherein the solvent used for dissolving phospholipids is ethanol.

23. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (22) wherein parenterally acceptable aqueous phase is water or phosphate buffer; in case micronised Amphotericin B is used, it is water, phosphate buffer, saline or phosphate buffer saline.

10

24. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (23) wherein the pH of said aqueous phase used for suspension of Amphotericin B at step (ii) is adjusted to 6.0 - 15 8.0.

25. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (24) wherein sterilisation of the 20 homogenised filtered suspension is carried out by conventional autoclaving.

26. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (25) wherein the sterilisation 25 temperature is 110°C.

27. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (26) wherein sterilisation is carried 30 out by specialised process of autoclaving in which the heating and cooling time is reduced by rapid heat and rapid cool cycle.

28. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (27) wherein when Amphotericin B used is micronised, said aqueous phase used in step (ii) is water, saline, phosphate buffer or phosphate buffer saline.

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29. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (13) - (28) wherein when Amphotericin B used is micronised, sodium chloride is added at any step (ii) to (iv) so that the sodium chloride content of the final product is atleast 0.1% w/v.

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30. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids as claimed in any of claims (1) - (12) prepared by the process as claimed in any of claims (13) - (29).

15

31. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids substantially as herein described in the Text and in the Examples I - XII of the invention.

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32. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids substantially as herein described in the Text and in the Examples I - XII of the invention.

25

33. A low toxicity parenteral dimethyl sulfoxide free aqueous composition containing Amphotericin B, sodium chloride and phospholipids prepared by the process substantially as herein described in the Text and in the Examples I - XII of the invention.

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34. A low toxicity parenteral dimethyl sulfoxide free and chlorinated hydrocarbon free aqueous composition containing Amphotericin B, sodium chloride and

phospholipids substantially as herein described in the Text and in the Example VII of the invention.

35. A process for manufacture of a low toxicity parenteral dimethyl sulfoxide free and chlorinated hydrocarbon free aqueous composition containing Amphotericin B, sodium chloride and phospholipids substantially as herein described in the Text and in the Example VII of the invention.
- 5
36. A low toxicity parenteral dimethyl sulfoxide free and chlorinated hydrocarbon free aqueous composition containing Amphotericin B, sodium chloride and phospholipids prepared by the process substantially as herein described in the Text and in the Example VII of the invention.
- 10

INTERNATIONAL SEARCH REPORT

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 418 153 A (MEDGENIX GROUP) 20 March 1991 (1991-03-20) claims 1-16 page 2, line 12 - line 14 page 3, line 6 - line 13 page 6, line 26 - line 35 example 1 -----	1-12



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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