

Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES

E. S. D.

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Re: **P1.-PHARMACIA ITALIA S.p.A Y PHARMACIA & UPJOHN COMPANY.-Solicitud de Patente de Invención en Fase Nacional PCT para "SUSPENSIÓN ACUOSA ESTABILIZADA PARA USO PARENTERAL"-Clase A61K 9/10.-Expediente número 02-102.352.-**

1. Me refiero a mi solicitud de patente del caso en referencia de 12 de Noviembre de 2002.

2. Atentamente me permito presentar a ese Despacho nuevas páginas 36, 37, 38, 39, 40, 41 y 42 de las reivindicaciones para reemplazar aquellas presentadas inicialmente, en las cuales se han realizado las siguientes modificaciones:

(a) Debidamente autorizado por mi representado se han cancelado las reivindicaciones 10 y 11 dirigidas a proteger el "uso":

(b) Las reivindicaciones restantes 1 a 9 han sido re-organizadas en ocho (8) reivindicaciones de la siguiente manera:

REIVINDICACIÓN NUEVA	SUSTENTO
1	1
2	5
3	Parte de la 6, página 13 de la descripción.
4	7
5	8
6	9
7	2

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8	4
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Es necesario llamar la atención al hecho de que la reivindicación 3 (original) corresponde a la nueva reivindicación 24, que me permito exponer a continuación; y

- (c) Se han incluido 26 nuevas reivindicaciones reenumeradas como nuevas reivindicaciones 9 a 34, dirigidas a proteger una formulación farmacéutica en suspensión acuosa preferida en la presente invención. El sustento para estas nuevas reivindicaciones se encuentra claramente establecido en las páginas 14 a 20 de la descripción. Particularmente, la nueva reivindicación 24 tiene su sustento a partir de la reivindicación 3 presentada originalmente.

Sobre, este particular, atentamente me permito manifestar a ese Despacho que las modificaciones propuestas **no amplían** de manera alguna el alcance de la presente invención, toda vez que las mismas solamente obedecen a la protección específica de realizaciones preferidas de la invención para las formulaciones reivindicadas, las cuales tienen su sustento en la descripción y en las reivindicaciones originales, como se demostró anteriormente.



3. Anexos:

- (a) Recibo No. 04-81.459, expedido por la Superintendencia de Industria y Comercio, que acredita el pago de la tasa de las modificaciones;
- (b) Copia de las páginas 36, 37, 38, 39, 40, 41 y 42 de las reivindicaciones que reemplazan las originalmente presentadas, en las cuales se han realizado las modificaciones expuestas anteriormente; y
- (c) Formulario único de correcciones y modificaciones.

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4. En vista de lo anterior, ruego a Usted tomar atenta nota y proceder de conformidad.

Señor Superintendente,



ALICIA LLOREDA RICAURTE

T.P. 53.215

Código 231

SECRETARIA SEXTA DE BOGOTÁ

CONOCIMIENTO Y PRESENTACION PERSONAL

E.C. 29 OCT 2004

JOAN MANUEL BOTERO MEDINA

SECRETARIO DEL CIRCULO DE BOGOTÁ

Recibí (señalar):

39.690.713

Yo, (señalar) que lo(s) firmante(s) aparece(n) en el presente documento es(son) la(s) su(s)ya(s) que el contenido del mismo es cierto. En constancia se firma esta diligencia.




ALICIA LLOREDA RICAURTE

C.C. No. 39.690.713 de Usaquén



FORMULARIO ÚNICO DE CORRECCIONES Y MODIFICACIONES.
2000-10

①

SOLICITUD DE:

☐

Corrección de Error Material.

☐

Modificación a una Solicitud.

②

SOLICITANTE

Nombre: **PHARMACIA & ITALIA S.p.A. y PHARMACIA & UPJOHN COMPANY**, sociedades existentes y organizadas de conformidad con las leyes de Italia y el Estado de Delaware, U.S.A.
Dirección: **Via Robert Koch, 1.2., I-20152 Milan, Italia y 301 Henrietta Street, Kalamazoo, Michigan 49001, Estados Unidos de América.**
Teléfono: **3 264270** **6 069701**
E-mail: **jllcco@lloredacamacho.com**
Domicilio: **Milan, Italia y Kalamazoo, Estado de Michigan, Estados Unidos de América**

IDENTIFICACION

C.C. ☐

NIT ☐

C.E. ☐

Otro ☐

Número _____

③

**REPRESENTANTE
O APODERADO**

Nombre: **ALICIA LLOREDA RICAURTE**
Dirección: **Calle 72 No. 5-83 Piso 5º, Bogotá - Colombia**
Teléfono: **3 264270** Fax: **2 126426**
E-mail: **jllcco@lloredacamacho.com**

IDENTIFICACION

C.C. ☐

NIT ☐

C.E. ☐

Otro ☐

Número **39.690.713** TP **53.215**

④

Número de expediente:

02-102.352

⑤

Descripción de la corrección o modificación solicitada modificación al capítulo reivindicatorio.

⑥

Comprobante de pago No. 04-81.459

Fecha 14 de Octubre de 2004

⑦

Anexos:

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Comprobante de pago de la tasa.

☐

Poderes, si fuere el caso.

☐

Certificado de existencia y representación legal de las partes, cuando sean personas jurídicas.

⑧

NOMBRE ALICIA LLOREDA RICAURTE

FIRMA



C.C. **39.690.713**

T.P. **53.215**

Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

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NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 04 - 81,459
FECHA : OCTUBRE 14 DE 2004

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
LLOPEDA Y CIA S. A	CONSIGNACION	BANCO POPULAR	050-00110-6	28713710	14/10/2004	1,122,000.

***** C O N C E P T O *****

CANT. HISTORICO	CONCEPTO	TOTAL	CONCEPTO
1	50005-01-03 CAMBIO DE MODALIDAD Y MODIFICA	12	MARCAS Y LENAS COMERCIALES
			TOTAL :
			87,000.00
			87,000.00

SON: OCHENTA Y SIETE MIL PESOS

RESPONSABLE : 

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

HA
|||REIVINDICACIONES

1. Una formulación en suspensión acuosa farmacéutica para su administración parenteral que tiene un pH sustancialmente estabilizado que comprende un compuesto biológicamente activo y una concentración efectiva de control del pH de L-metionina.

2. Una formulación farmacéutica de acuerdo con la reivindicación 1, caracterizada porque la concentración efectiva de control del pH de L-metionina está comprendida entre aproximadamente 0,005% p/v o p/p a (aproximadamente) 5% p/v o p/p.

3. Una formulación en suspensión acuosa farmacéutica para su administración parenteral que tiene un pH sustancialmente estabilizado, que comprende un compuesto biológicamente activo, un agente buffer y L-metionina en concentraciones efectivas para producir un efecto superaditivo de control del pH.

4. Una formulación farmacéutica de acuerdo con la reivindicación 3, caracterizada porque el agente de buffer es una sal de ácido fosfórico en una concentración inferior a 0,4% p/v o p/p.

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5. Una formulación farmacéutica de acuerdo con la reivindicación 4, caracterizada porque la concentración de las sales de ácido fosfórico es inferior a 0,2% p/v o p/p.

6. Una formulación farmacéutica de acuerdo con cualquiera de las reivindicaciones anteriores, caracterizada porque el rango de pH de la formulación está comprendido entre aproximadamente 3,0 a aproximadamente 8,0.

7. Una formulación farmacéutica de acuerdo con la reivindicación 6, caracterizada porque el compuesto biológicamente activo es un compuesto esteroidal.

8. Una formulación farmacéutica de acuerdo con la reivindicación 7, caracterizada porque el compuesto esteroidal biológicamente activo se selecciona entre exemestano, medroxiprogesterona acetato y estradiol cipionato o una mezcla de medroxiprogesterona acetato y estradiol cipionato.

9. Una formulación en suspensión acuosa farmacéutica para su administración parenteral, que tiene un pH sustancialmente estabilizado con un pH desde aproximadamente 3.0 a aproximadamente 8.0, que comprende un compuesto biológicamente activo;

L-metionina en aproximadamente 0.005% p/v a aproximadamente 5% p/v, y opcionalmente uno o más de un tensoactivo; un agente de suspensión; un agente espesante; un agente quelante de metal, antioxidante; un compuesto que contiene

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tiol; un preservativo; un agente de ajuste de la tonicidad; y/o un agente buffer convencional, en aproximadamente 5% a aproximadamente 25%.

10. Una formulación farmacéutica de acuerdo con la reivindicación 9, caracterizada porque la L-metionina está presente en aproximadamente 0.01% p/v a aproximadamente 1.0% p/v.

11. Una formulación farmacéutica de acuerdo con la reivindicación 9, caracterizada porque incluye un agente de suspensión seleccionado de una polivinilpirrolidona y un polietilen glicol y combinaciones de los mismos.

12. Una formulación farmacéutica de acuerdo con la reivindicación 11, caracterizada porque incluye polietilen glicol con un peso molecular desde aproximadamente 300 a aproximadamente 6000.

13. Una formulación farmacéutica de acuerdo con la reivindicación 11, caracterizada porque incluye polivinilpirrolidona con un peso molecular desde aproximadamente 7000 a aproximadamente 54000. ✓

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14. Una formulación farmacéutica de acuerdo con la reivindicación 13, caracterizada porque la polivinilpirrolidona es PVP K12 o K17.

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15. Una formulación farmacéutica de acuerdo con la reivindicación 14, caracterizada porque la polivinilpirrolidona es PVP K17.

16. Una formulación farmacéutica de acuerdo con la reivindicación 9, caracterizada porque incluye un agente de buffer convencional seleccionado de un buffer fosfato y citrato.

17. Una formulación farmacéutica de acuerdo con la reivindicación 16, caracterizada porque el agente buffer es una sal de ácido fosfórico presente en menos de 0.4% p/p o p/v.

18. Una formulación farmacéutica de acuerdo con la reivindicación 17, caracterizada porque la sal del ácido fosfórico está presente en menos de 0.2% p/p o p/v.

19. Una formulación farmacéutica de acuerdo con la reivindicación 17, caracterizada porque el agente buffer es fosfato de sodio.

20. Una formulación farmacéutica de acuerdo con la reivindicación 9, caracterizada porque el pH es de aproximadamente 3.0 a aproximadamente 7.5.

21. Una formulación farmacéutica de acuerdo con la reivindicación 20, caracterizada porque el pH es de aproximadamente 4.0 a aproximadamente 7.0.

22. Una formulación farmacéutica de acuerdo con la reivindicación 9, caracterizada porque el compuesto biológicamente activo es un compuesto esteroideal.

23. Una formulación farmacéutica de acuerdo con la reivindicación 22, caracterizada porque el compuesto esteroideal está en forma molida o micronizada.

24. Una formulación farmacéutica de acuerdo con la reivindicación 22, caracterizada porque el compuesto esteroideal biológicamente activo se selecciona entre medroxiprogesterona acetato, exemestano, estradiol cipionato, metilprednisolon acetato, oxabolon cipionato, clostebol acetato y testosterona cipionato.

25. Una formulación farmacéutica de acuerdo con la reivindicación 24, caracterizada porque el compuesto esteroideal biológicamente activo se selecciona entre medroxiprogesterona acetato, exemestano, estradiol cipionato y una combinación de dos o más de los mismos.

26. Una formulación farmacéutica de acuerdo con la reivindicación 25, caracterizada porque el compuesto esteroideal biológicamente activo es medroxiprogesterona acetato y está presente desde aproximadamente 1% p/v a aproximadamente 40% p/v.

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27. Una formulación farmacéutica de acuerdo con la reivindicación 26, caracterizada porque la medroxiprogesterona acetato está presente desde aproximadamente 3% p/v a aproximadamente 30% p/v.

28. Una formulación farmacéutica de acuerdo con la reivindicación 25, caracterizada porque el compuesto esteroidal biológicamente activo es estradiol cipionato y está presente desde aproximadamente 0.1% p/v a aproximadamente 5% p/v.

29. Una formulación farmacéutica de acuerdo con la reivindicación 28, caracterizada porque el (estradiol) cipionato está presente desde aproximadamente 0.25% p/v a aproximadamente 2.5% p/v.

30. Una formulación farmacéutica de acuerdo con la reivindicación 25, caracterizada porque la medroxiprogesterona acetato está presente desde aproximadamente 3% p/v a aproximadamente 30% p/v, y el estradiol cipionato está presente desde aproximadamente 0.1% p/v a aproximadamente 5% p/v.

31. Una formulación farmacéutica de acuerdo con la reivindicación 25, caracterizada porque el compuesto esteroidal biológicamente activo es exemestano y está presente desde aproximadamente 1% p/v a aproximadamente 25% p/v.

32. Una formulación farmacéutica de acuerdo con la reivindicación 31, caracterizada porque el exemestano está presente desde aproximadamente 5% p/v a aproximadamente 20% p/v. 11Y

33. Una formulación farmacéutica de acuerdo con la reivindicación 9 adecuada para administración intramuscular o subcutánea. ?
Ac. D. de glicol

34. Un vial que contiene una formulación de acuerdo con cualquiera de las reivindicaciones anteriores, con un manto de nitrógeno en el espacio superior del vial. ?

DIVISION DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 02-102352

**EL EXTRACTO DE LA PRESENTE SOLICITUD DE PATENTE DE INVENCION -
PCT FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No 532,
DE FECHA 30 DE SEPTIEMBRE DE 2003, EL TERMINO HABIL SEÑALADO
POR LA LEY EXPIRA EL 30 DE DICIEMBRE DE 2003.**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud calga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI

NO **X**

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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES**

2020

Bogotá, D.C., 01 de marzo de 2007

Apoderado: Alicia Lloreda Ricaurte

Oficio: 3662

REFERENCIA: Radicación No. 02-102352
Trámite 002
Evento 001
Actuación 430
Folios

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados:

- ◆Capítulo descriptivo, folios 42 a 76;
- ◆Capítulo reivindicatorio modificado, 114 a 120;
- ◆Solicitud PCT/US01/04643 en fase nacional;
- ◆Solicitud del examen de patentabilidad, folios 97 y 98.

Objeto de la invención:

La presente solicitud cuyo título es "SUSPENSIÓN ACUOSA ESTABILIZADA PARA USO PARENTERAL"

El objeto de la solicitud es composiciones que comprenden una concentración de L-metionina.

El objeto de la invención se puede clasificar según la clasificación Internacional como A61K 47/18

Claridad de la solicitud:

En el artículo 30 de la decisión 486 de la Comisión de la Comunidad Andina se establece: *Las reivindicaciones definirán la materia que se desea proteger mediante la patente. Deben ser claras y concisas y estar enteramente sustentadas por la descripción.*

Las reivindicaciones no deben contener expresiones vagas tales como *"tiene un pH sustancialmente estabilizado"*, *"una concentración efectiva del control de pH"*, *"en concentraciones efectivas para producir un efecto superaditivo de control de pH"*, para definir rangos de cantidades se introduce la expresión *"desde aproximadamente...hasta aproximadamente"* para restarle claridad, no es claro porque cualquier composición se caracteriza por sus elementos constitutivos y cuando se definen rangos debe ser así, definir claramente los límites, tal como lo indica la norma, perfectamente delimitado.

-La reivindicación 34, que reclama un vial el cual no está descrito como tal, porque los viales son recipientes generalmente de vidrio con características particulares, o de materiales especiales, no están descritos como tal y son de otro ámbito tecnológico, y mucho menos se puede aceptar que se pueda caracterizar por el contenido.

-La reivindicación 33, se encabeza como una formulación ya reivindicada, pero se caracteriza no por su composición como tal, sino por la forma final que se desean que tenga.

Determinación del estado de la técnica:

El artículo 16 de la Decisión 486 de la Comisión establece: *"El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida."*

Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40."

N	Documento No.	Título	Fecha de Publicación
D1	US5.589.484	Stable aqueous dispersions containing liposomes	29-oct-1996
D2	WO9811912	Pharmaceutical formulations of corticotropin releasing factor having improved stability in liquid form	26-mar-1998
D3	WO9915193	Liquid interferon- β formulation	01-abr-1999

Evaluación de los requisitos de patentabilidad:

El artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina establece: *"Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial."*

En cumplimiento de este artículo, se procedió a evaluar los requisitos de novedad, nivel inventivo y aplicación industrial con el fin de establecer la patentabilidad de la invención reivindicada en la solicitud en estudio.

Evaluación de la Novedad:

El Art.16 de la Decisión 486 de la Comisión de la Comunidad Andina establece: *"Una invención se considerará nueva cuando no está comprendida en el estado de la técnica." "El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida".*

La solicitud en estudio reclama como nueva una suspensión acuosa farmacéutica para su administración parenteral que tiene un pH sustancialmente estabilizado que comprende un compuesto biológicamente activo y una concentración efectiva de control de pH de L-metionina.

-La anterioridad D1 describe composiciones en dispersiones acuosas que comprenden como un principio activo que puede ser una hormona, un agente estabilizante metionina, buffer de fosfato para el control efectivo del pH, cloruro de sodio para isotonzar la suspensión que se va a aplicar. Entonces lo que se observa es que en la presente solicitud en estudio, se hizo una generalización de lo enseñado en esta anterioridad, en las reivindicaciones generales.

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-La anterioridad D2 describe formulaciones farmacéuticas que comprenden un buffer de que estabiliza el pH de acuerdo con la necesidad y va en el rango de 2,0 a 5,0, o de de 6,0 a 9,0; el buffer puede ser de fosfato o citrato por ejemplo; también comprende un aminoácido que incluye metionina, en concentración de un rango de 1mM a 100mM; junto con un principio activo y todos los auxiliares normalmente utilizados para obtener la composición farmacéutica; lo que indica que la materia reclamada como nueva es del estado de la técnica, porque no es la primera vez en el estado de la técnica que se encuentran en una formulación metionina, que el pH sustancialmente estabilizado y que contenga un principio activo, tal como puede ser una hormona.

-La anterioridad D3 describe formulaciones farmacéuticas para uso parenteral, que comprenden un buffer que puede mantener el pH estable en un rango entre 5 a 8, se puede seleccionar de buffer fosfato o citrato; metionina; un principio activo. y con vehículos farmacéuticamente aceptables de uso común para adaptarlos a la forma farmacéutica deseable. Se puede ver que D3 destruye categóricamente la novedad la solicitud en estudio, puesto que esta anterioridad enseña la misma mezcla en que la presente solicitud basa la novedad; por lo tanto "la formulación" que se reclama como nuevo en la solicitud donde la novedad de basa en la presencia de metionina en una composición con pH sustancialmente estable y tal como se puede ver esa mezcla ya era conocida en el estado de la técnica.

Evaluación del Nivel Inventivo:

El art. 18 de la Decisión 486 de la Comisión de la Comunidad Andina establece: ***"Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica."***

La presente solicitud parece no tener nivel, ya que tanto D1 a D3 como la solicitud en estudio se refieren a composiciones que comprendan metionina, en una composición que este el pH sustancialmente estabilizado, que las tres anterioridades comprenden esos elementos que son la base de la novedad de la presente solicitud, la diferencia esta en el nombre específico de los esteroides, aunque las tres anterioridades contemplan la posibilidad, que el principio activo sea un esteroide, o sea la única diferencia están en los nombres específicos de los esteroides; entonces cuando observamos el efecto de la combinación en que

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se basa la novedad de la solicitud en estudio contra el de D1 a D3, notamos que no se demuestra la ventaja técnica real, por lo tanto se considera que dicha modificación es obvia para una persona normalmente versada en la materia, con ligera modificación en las composiciones originales, solo al establecer nombres específicos en los compuestos esteroides. En consecuencia esta oficina considera que la solicitud en estudio no cumple con el requisito de nivel inventivo.

Se debe tener claro que cualquier solicitud afectada por novedad, menos tiene nivel inventivo.

No.	Documento	Reivindicaciones Afectadas	Requisito que afecta
D1	US5.569.464	1-10, 16-24; 25 a 32	Novedad Nivel inventivo
D2	WO9811912	1-10, 16-24 25 a 32	Novedad Nivel inventivo
D3	WO9915193	1-10, 16-24; 25 a 32	Novedad Nivel inventivo

En cumplimiento del artículo 45 de la decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta días contados a partir de la fecha de la notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo 5° del título 1° de la Circular Única n° 10 de 2001.

ALIX CARMENZA CÉSPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones

23 MAR. 2007

El anterior requerimiento fue notificado por fijación en lista de fecha_____.

CONCEPTO TÉCNICO No. 437	EXAMINADOR TÉCNICO Código: 202012
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D1.

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US005569464A

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United States Patent [19]
Endo et al.

[11] **Patent Number:** **5,569,464**
[45] **Date of Patent:** **Oct. 29, 1996**

[54] **STABLE AQUEOUS DISPERSIONS
CONTAINING LIPOSOMES**

[75] **Inventors:** Kenji Endo, Fujisawa; Hidekazu
Suzuki, Kanagawa-ken; Tetsu Oguma,
Hadano; Masayoshi Goto, Tokyo, all of
Japan

[73] **Assignee:** Wakamoto Pharmaceutical Co., Ltd.,
Tokyo, Japan

[21] **Appl. No.:** 448,972

[22] **Filed:** May 24, 1995

Related U.S. Application Data

[63] **Continuation of Ser. No. 216,854, Mar. 24, 1994, aban-**
doned.

[30] **Foreign Application Priority Data**

Apr. 2, 1993 [JP] Japan 5-098367

[51] **Int. Cl.⁶** A61K 9/127

[52] **U.S. Cl.** 424/450; 428/402.2

[58] **Field of Search** 424/450; 428/402.2

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,946,683 8/1990 Forson 424/422

FOREIGN PATENT DOCUMENTS

1280975	3/1991	Canada .
0211647	2/1987	European Pat. Off. .
0467275	1/1992	European Pat. Off. .
0472225	2/1992	European Pat. Off. .
2660192	10/1991	France .
0046921	3/1982	Japan .
2-273339	11/1990	Japan .
2-295917	12/1990	Japan .
2103485	2/1983	United Kingdom .

OTHER PUBLICATIONS

Abstract of Japanese patent application JP2273539, Nov.
1990, Derwent Publications Ltd.

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[57] **ABSTRACT**

An aqueous dispersion containing liposomes comprising
yolk lecithin and/or soybean lecithin as lipids for forming
liposomes wherein the dispersion contains a hydroxy acid
and an amino acid, which hardly shows coloration, shows
little leak of drugs encapsulated in the liposomes and is
stable in a broad pH range.

7 Claims, No Drawings

Among these amino acids, methionine, histidine, arginine, glutamic acid, aspartic acid and their pharmaceutically acceptable salts are particularly preferred.

The essential characteristics of the present invention reside in the use of at least one of the above hydroxy acids and at least one of the above amino acids in combination. Therefore, several kinds of the above hydroxy acids and several kinds of the above amino acids may be used in combination, and it is not intended to exclude using mixtures of more than two kinds of the compounds.

Furthermore, the concentration ranges of the hydroxy acids and the amino acids where the advantages can be obtained may be somewhat varied depending on the kinds of the hydroxy acid and the amino acid used in combination.

For example, when sodium citrate and methionine are used, from 0.05 to 5 parts by weight of sodium citrate (in terms of citric acid) and from 0.008 to 0.8 parts by weight of methionine can be suitably used with a part by weight of lipids for forming liposomes.

When sodium citrate and histidine hydrochloride are used, from 0.05 to 5 parts by weight of sodium citrate (in terms of citric acid) and from 0.0006 to 0.3 parts by weight of histidine hydrochloride (in terms of histidine) can be suitably used with a part by weight of lipids for forming liposomes.

When sodium citrate and arginine are used, from 0.05 to 5 parts by weight of sodium citrate (in terms of citric acid) and from 0.02 to 4 parts by weight of arginine can be suitably used with a part by weight of lipids for forming liposomes.

When sodium citrate and sodium glutamate or sodium aspartate are used, from 0.05 to 5 parts by weight of sodium citrate (in terms of citric acid) and from 0.015 to 3 parts by weight of sodium glutamate or sodium aspartate (in terms of glutamic acid or aspartic acid) can be suitably used with a part by weight of lipids for forming liposomes.

The advantages of the present invention can be attained by using a hydroxy acid and an amino acid in combination in amounts within the above proportion with respect to lipids for forming liposomes. On the other hand, when the amount of the these stabilizing agents in an aqueous dispersion containing liposomes is greater than a certain amount, the operability of the dispersion will be declined because of the increase of viscosity of the dispersion and phase separation will be occurred because of floating of liposomes and they are not preferred. Moreover, when the amount of the amino acid exceeds a certain amount, decrease of the stabilizing effect is observed. Therefore, for a part by weight of an aqueous dispersion containing liposomes, it is normally preferred that not more than 0.13 parts by weight of the hydroxy acid and, while the upper limit of the amount of the amino acid is varied depending on its kind, not more than 0.02 parts by weight of methionine, 0.015 parts by weight of histidine, 0.05 parts by weight of arginine or 0.08 parts by weight of glutamic acid or aspartic acid are used.

The drugs to be contained in the liposomes of the present invention are not particularly limited and may be either hydrophilic or lipophilic. For example, they may be agents affecting central nervous system, agents affecting peripheral nervous system, cardiovascular agents, agents affecting respiratory organs, agents affecting digestive organs, hormone preparations, vitamin preparations, agents related to blood and body fluids, agents affecting metabolism, carcinostatic agents, antiallergic agents, antibiotics preparations, chemotherapeutics, agents for ophthalmic use, diagnostic agents, analgesics and sedatives agents, physiologically active sub-

stances, prostaglandin agents, immunoregulatory drugs, anti-inflammatory agents and the like. Examples of these agents are set forth below, but the present invention is not limited to them:

- (1) agents affecting central nervous system such as:
 - a) hypnotics and sedatives and antianxiety agents such as alprazolam and diazepam;
 - b) antiepileptics such as phenobarbital;
 - c) antipyretics, analgesics and antiinflammatory agent such as paracetamol and ketoprofen;
 - d) psychotropic agents such as amitriptyline and chlorpromazine;
- (2) agents affecting peripheral nervous system such as:
 - a) local anesthetics such as lidocaine, benzocaine, tetracaine and dibucaine;
 - b) antispasmodics such as atropine and atfoqualone;
- (3) cardiovascular agents such as:
 - a) antihypertensives such as reserpine and hydralazine;
 - b) vasoconstrictors such as phenylephrine, naphazoline and methoxamine;
 - c) vasodilators such as diltiazem and nifedipine; d) anti-hyperlipemia agents such as clofibrate and pravastatin;
- (4) agents affecting respiratory organs such as:
 - a) antitussives such as methylephedrine and ephedrine;
 - b) bronchodilators such as theophylline; and pharmaceutically acceptable salts thereof.
- (5) agents affecting digestive organs such as:
 - a) antilicer agents such as farnesol, geraniol and 2-N-[3-[3-(1-piperidinomethyl)phenoxy] propyl]amino-5-amino-1,3,4-thiadiazole (hereinafter abbreviated as "TAS");
 - (6) hormone preparations such as:
 - a) adrenal hormone preparations such as betamethasone phosphate, dexamethasone phosphate, hydrocortisone, triamcinolone and prednisolone;
 - b) hormone preparations such as insulin;
- (7) vitamin preparations such as vitamin A and preparations and vitamin C and preparations;
- (8) agents related to blood and body fluids such as:
 - a) hemostatics such as carbazochrome, tranexamic acid;
- (9) agents affecting metabolism such as:
 - a) agents for liver disease such as glycynithic acid-DL-methionine;
 - b) enzyme preparations such as superoxide dismutase, urokinase and t-PA
 - c) agents for diabetic complications such as epalrestat;
- (10) carcinostatic agents such as mitomycin, adriamycin, methotrexate, cisplatin, tegafur, vincristine and doxorubicin;
- (11) antiallergic agents such as:
 - a) antihistamines such as chlorpheniramine and diphenhydramine;
 - b) antiallergic agents such as cromoglicic acid, trimast and ketotifen;
- (12) antibiotics preparations such as amphotericin B, gentamicin, erythromycin, tetracycline, chloramphenicol and colistin methanesulfonic acid;
- (13) chemotherapeutics such as ofloxacin, norfloxacin and miconazole;
- (14) agents for ophthalmic use such as:
 - a) antiglaucoma drugs such as epinephrine, timolol and carteolol;
 - b) anticataract drugs such as glutathione and phenoxazine;

- c) mydriatics and preparations such as tropicamide;
- d) miotics and preparations such as pilocarpine and carbachol;
- (15) diagnostic agents such as fluorescein;
- (16) analgesics and sedatives agents such as morphine and codeine;
- (17) physiologically active substances such as erythropoietin;
- (18) prostaglandin agents such as prostaglandin E₁ and prostaglandin A₁;
- (19) immunoregulatory drug such as interferon, interleukin, muramyl dipeptide, muramyl tripeptide, penicillamine and ciclosporin;
- (20) anti-inflammatory agents such as fluorometholone, glycyrrhizic acid and diclofenac;

As examples of the "pharmaceutically acceptable salts" of the hydroxy acids, the amino acids and the drugs used for the present invention, sodium salts, potassium salts and the like can be mentioned for the hydroxy acids, glutamic acid, diclofenac, betamethasone phosphate and the like, hydrochloride salts, sulfate salts and the like can be mentioned for arginine, histidine, gentamicin, morphine, lidocaine, pilocarpine and the like, and maleate salts and the like can be mentioned for chlorpheniramine and timolol.

The process for preparing the aqueous dispersion containing liposomes of the present invention will be explained. Though various known methods are applicable, it can be prepared, for example, as follows. The lipids for forming liposomes comprising yolk lecithin and, if desired, cholesterol and the like as membrane-forming adjuvants are dissolved in a suitable solvent such as chloroform and the solvent is evaporated in vacuo to prepare a lipid film. This film is suspended in water, and a hydroxy acid and an amino acid are added to give an aqueous dispersion containing liposomes of the present invention. Alternatively, the lipid film prepared as above can be suspended in a solution containing a hydroxy acid and an amino acid to give an aqueous dispersion containing liposomes of the present invention. Moreover, an aqueous dispersion containing liposomes encapsulating drugs according to the present invention can be prepared as follows. When the drug is a hydrophilic drug, the drug may be dissolved in water or a solution containing a hydroxy acid and an amino acid for suspending the lipid film, and the liposomes may be prepared as the same manner as above. When the drug is a lipophilic drug, the drug may be mixed in the organic solvent with lipids for forming liposomes and, if desired, membrane-forming adjuvants and the liposomes can be prepared as the same manner as above. With respect to the aqueous dispersion containing liposomes obtained as above, if necessary, encapsulation efficiency of drugs can be increased by repeating freeze-thawing process, or the size distribution of the liposomes can be controlled by the extrusion method (M. J. Hope, M. B. Bally, G. Webb and P. R. Cullis, *Biochim. Biophys. Acta* 812, 55 (1985)) or the like. The drugs which are not encapsulated in the liposomes can be removed by gel filtration, centrifugal separation, affinity chromatography or the like.

The uses of the aqueous dispersion containing liposomes obtained as described above are expected in various applications and it may be used, for example, as cosmetics such as moisture-holding agents for skin care and as pharmaceutical preparations such as parenteral injections, eye drops, nasal drops, endermic agents, inhalation agents and oral agents.

In preparing the aqueous dispersion containing liposomes of the present invention as formulations such as those

described above, conventional additives such as isotonic agents, pH adjusting agents, antiseptic agents and stabilizing agents may be added so long as the advantages of the dispersion according to the present invention are not deteriorated. Moreover, the aqueous dispersion containing liposomes of the present invention may be subjected to a sterilizing treatment by a fractional sterilization method. The stability of the dispersion is not decreased by this treatment. Furthermore, as a method for preventing oxidation of parenteral injection and the like, nitrogen gas substitution is generally employed when a liquid preparation is filled in ampouls and the like. Though the aqueous dispersion containing liposomes of the present invention is stable without the nitrogen gas substitution, a greater stability can be expected by employing such nitrogen gas substitution.

EXAMPLES

The present invention will be further illustrated by referring to the following examples, but it is not intended to restrict the present invention to the examples. The term "percent (%)" used in the following examples means "percent by weight" excepting those used for encapsulation efficiency and drug-remaining ratio.

Stability of dispersion was evaluated by determining degree of coloration, relative encapsulation efficiency and drug-remaining ratio. The degree of apparent coloration was evaluated by determining color difference (ΔE) between samples before and after storage by means of a color difference meter (Model ND-504 DR: Nihon Densyoku Industry Co., Ltd.). Coordinate values based on the UCS system of color representation, L , a and b , were measured by the color difference meter with respect to the same samples before and after the storage, and the values of ΔE were calculated in accordance with the following equation:

$$\Delta E = \sqrt{(L_1 - L_2)^2 + (a_1 - a_2)^2 + (b_1 - b_2)^2} / 12$$

In the above equation, L_1 , a_1 and b_1 are the measured values obtained for a sample before storage and L_2 , a_2 and b_2 are for a sample after storage.

The ΔE values obtained in the following examples were classified into the 5 ranges shown below and represented by the appended term or symbols as the evaluation of appearance.

- No change: $\Delta E < 2.5$
- * (Faint yellow): $2.5 \leq \Delta E < 3.5$
- ** (Light yellow): $3.5 \leq \Delta E < 5.0$
- *** (Yellow): $5.0 \leq \Delta E < 8.0$
- **** (Yellowish brown): $\Delta E \geq 8.0$

The relative encapsulation efficiency (%) and the drug-remaining ratio (%) were calculated in accordance with the following equations, respectively:

Relative encapsulation efficiency (%) =
$$\frac{\text{Encapsulation efficiency after storage (\%)}}{\text{Encapsulation efficiency before storage (\%)}} \times 100$$

Drug-remaining ratio (%) =
$$\frac{\text{Total drug concentration after storage}}{\text{Total drug concentration before storage}} \times 100$$

In the above equations, the "total drug concentration" is obtained as follows. First, a drug concentration of liquid obtained by treating an aqueous dispersion containing liposomes with Triton X-100 to destroy liposomes contained in

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the dispersion is determined. Then, the total drug concentration is calculated from the above-obtained drug concentration of the liquid so that the total drug concentration expresses the drug concentration in the original dispersion. Further, the "encapsulation efficiency" was obtained in accordance with the following equation:

Encapsulation efficiency (%) =

$$\frac{\text{Total drug concentration} - \text{Unencapsulated drug concentration}}{\text{Total drug concentration}} \times 100$$

In the above equation, the term "total drug concentration" has the same meaning as above and the term "unencapsulated drug concentration" is determined as a drug concentration in a filtrate obtained from ultrafiltration of an aqueous dispersion for removing the liposomes.

Example 1

0.25 g of egg phosphatidylcholine (PC purity: more than 95%, Nihon Yushi Co., Ltd.) and 0.125 g of cholesterol were dissolved in 20 ml of a chloroform/methanol mixture (10:1) and the solvent was removed by an evaporator to form a lipid film.

5 ml of phosphate buffer of pH 7.4 (made isotonic with sodium chloride) was added to this lipid film and strongly vortexed, and the obtained liquid was extruded under pressure through a polycarbonate membrane filter having a pore size of 0.2 μm (NUCLEOPORE®: available from Nomura

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Microscience Co., Ltd.) to obtain a milk white liposome stock dispersion of which liposomes contained egg phosphatidylcholine. Moreover, a milk white liposome stock dispersion of which liposomes contained soybean lecithin (phosphatidylcholine) was obtained in a manner similar to the above by using soybean lecithin (PC purity: 97.3%, Nihon Seika Co., Ltd.) instead of the egg phosphatidylcholine.

To one part by volume of the obtained liposome stock dispersions, one part by volume of a solution containing a hydroxy acid and one or more amino acids selected from various hydroxy acids and amino acids was added to obtain aqueous dispersions containing liposomes of the present invention. For comparison, to one part by volume of the above liposome stock dispersions, one part by volume of phosphate buffer containing a hydroxy acid or an amino acid alone, or one part by volume of phosphate buffer containing no stabilizing agents was added to give comparative aqueous dispersions containing liposomes.

These dispersions were filled into 1 ml glass ampoules and stored at 60° C. to be examined the change of their appearance. The changes of appearance after one-week storage are shown in Table 1. When any stabilizing agents (hydroxy acids and amino acids) are not used, or when a hydroxy acid or an amino acid is used alone, coloration was observed. On the other hand, the aqueous dispersions containing liposomes according to the present invention hardly exhibited change of appearance as compared with the dispersions before storage and therefore it was found that the aqueous dispersions containing liposomes according to the present invention were stable.

TABLE 1

Lipid for forming	Stabilizing agents ²⁾ (part by weight)		Appearance ³⁾
Phosphorus ¹⁾	Hydroxy acid	Amino acid	note ³⁾
<u>Present invention</u>			
Yolk lecithin	Lactic acid (0.08)	Sodium glutamate (0.06)	No change
Yolk lecithin	Malic acid (0.08)	Sodium glutamate (0.06)	No change
Yolk lecithin	Tartaric acid (0.08)	Sodium glutamate (0.06)	No change
Yolk lecithin	Sodium citrate (0.05)	Sodium glutamate (0.06)	No change
Yolk ⁷⁾ lecithin	Malic acid (0.08)	Methionine (0.06)	No change
Yolk lecithin	Malic acid (0.08)	Histidine hydrochloride (0.06)	*
Yolk lecithin	Malic acid (0.08)	Serine (0.08)	*
Yolk lecithin	Malic acid (0.08)	Arginine (0.08)	No change
Yolk lecithin	Malic acid (0.08)	Sodium aspartate (0.06)	No change
Yolk lecithin	Sodium citrate (0.05)	Methionine (0.06)	No change
Yolk lecithin	Sodium citrate (0.05)	Histidine hydrochloride (0.06)	No change
Yolk lecithin	Sodium citrate (0.05)	Serine (0.08)	No change
Yolk lecithin	Sodium citrate (0.05)	Arginine (0.08)	No change
Yolk lecithin	Sodium citrate (0.05)	Sodium aspartate (0.06)	No change
Yolk lecithin	Sodium citrate (0.05)	Methionine ⁷⁾ + Sodium aspartate (0.04 + 0.03)	No change
Yolk lecithin	Sodium citrate (0.05)	Methionine + Arginine (0.04 + 0.04)	No change
Yolk lecithin	Sodium citrate (0.05)	Methionine + Histidine hydrochloride (0.04 + 0.03)	No change

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TABLE 1-continued

Lipid for forming liposomes ^D	Stabilizing agents ^D (part by weight)		Appearance ^D
	Hydroxy acid	Amino acid	
Yolk lecithin	Sodium citrate (0.05)	Sodium aspartate + Arginine (0.03 + 0.04)	No change
Yolk lecithin	Sodium citrate (0.05)	Sodium aspartate + Histidine hydrochloride (0.03 + 0.03)	No change
Yolk lecithin	Sodium citrate (0.05)	Arginine + Histidine hydrochloride (0.04 + 0.03)	No change
Soybean lecithin	Sodium citrate (0.05)	Sodium aspartate (0.05)	No change
Comparative examples			
Yolk lecithin	—	—	****
Yolk lecithin	Lactic acid (0.16)	—	****
Yolk lecithin	Malic acid (0.16)	—	****
Yolk lecithin	Tartaric acid (0.16)	—	****
Yolk lecithin	Sodium citrate (0.1)	—	*
Yolk lecithin	—	Methionine (0.16)	**
Yolk lecithin	—	Histidine hydrochloride (0.12)	***
Yolk lecithin	—	Serine (0.16)	***
Yolk lecithin	—	Arginine (0.12)	**
Yolk lecithin	—	Sodium aspartate (0.12)	**
Yolk lecithin	—	Sodium glutamate (0.12)	*
Soybean lecithin	—	—	****
Soybean lecithin	Sodium citrate (0.1)	—	*
Soybean lecithin	—	Sodium aspartate (0.12)	**

^DThe concentration of the lipids for forming liposomes was 25 mg/ml.
^DThe amounts of the added stabilizing agents are shown in terms of parts by weight based on a part by weight of the lipids for forming liposomes. The amounts of the salts are represented by the amounts calculated as their free forms.
^DAppearance was examined after one-week storage at 60° C.
*: Faint yellow, **: Light yellow, ***: Yellow, ****: Yellowish brown

Example 2

To one part by volume of the liposome stock dispersion containing egg phosphatidylcholine obtained in Example 1, one part by volume of a solution containing sodium citrate and methionine, histidine hydrochloride, arginine or sodium glutamate at various concentrations was added to give aqueous dispersions containing liposomes, of which concentrations of the stabilizing agents are shown in Table 2.

These dispersions were stored at 60° C. and their changes of appearance were examined in a manner similar to Example 1. The changes of appearance after two-week storage were shown in Table 2. It was found that the advantage of the present invention is effectively obtained by using, with one part by weight of the lipids for forming liposomes, 0.05 to 5 parts by weight of citric acid together with one of the amino acids of the following amounts; 0.008 to 0.8 parts by weight for methionine, 0.0006 to 0.3 parts by weight for histidine, 0.02 to 2 parts by weight for arginine and 0.015 to 3 parts by weight for glutamic acid.

TABLE 2

<u>Stabilizing Agent¹³ (part by weight)</u>		
<u>Sodium citrate</u>	<u>Amino acid</u>	<u>Appearance²</u>
	<u>Methionine</u>	
0.05	0.8	*
0.05	0.08	No change
0.05	0.008	*
0.05	0.004	**
0.05	0	****
	<u>Histidine hydrochloride</u>	
0.05	0.6	**
0.05	0.3	*
0.05	0.0006	*
0.05	0.0003	**

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TABLE 2-continued

Stabilizing Agent ¹⁾ (part by weight)		
Sodium citrate	Amino acid	Appearance ²⁾
Arginine		
0.05	4	**
0.05	2	*
0.05	0.02	*
0.05	0.008	***
Sodium glutamate		
0.05	6	**
0.05	3	*
0.05	0.015	*
0.05	0.006	***
Methionine		
5	0.08	No change
0.05	0.08	No change
0.025	0.08	**
0	0.08	***
0	0	****
Histidine hydrochloride		
5	0.06	No change
0.05	0.06	No change
0.005	0.06	**
0	0.06	***
Arginine		
5	0.08	No change
0.05	0.08	*
0.005	0.08	**
0	0.08	***
Sodium glutamate		
5	0.06	No change
0.05	0.06	No change
0.005	0.06	**
0	0.06	***

¹⁾The amounts of the added stabilizing agents are shown in terms of parts by weight based on a part by weight of the lipids for forming liposomes. The amounts of the salts are represented by the amounts calculated as their free forms. (The concentration of the lipids for forming liposomes was 25 mg/ml.)
²⁾Appearance was examined after two-week storage at 60° C.
*: Faint yellow, **: Light yellow, ***: Yellow, ****: Yellowish brown

Example 3

Various lipids for forming liposomes shown in Table 3 were dissolved in a chloroform/methanol mixture (10:1) and the solvent was removed by an evaporator to form lipid films having various compositions.

5 ml of Britton-Robinson buffer of pH 7.0 was added to each film and strongly vortexed, and the obtained liquids were extruded under pressure through a polycarbonate membrane filter having a pore size of 0.2 μm (NUCLEOPORE®: available from Nomura Microscience Co., Ltd.) to give liposome stock dispersions having various lipid compositions.

One part by volume of a solution containing 4% of sodium citrate and 0.4% of sodium glutamate was added to one part by volume of the liposome stock dispersions obtained above to give aqueous dispersions containing liposomes according to the present invention. For comparison, one part by volume of the Britton-Robinson buffer was added to one part by volume of each of the above liposome stock dispersions to give comparative aqueous dispersions containing liposomes.

These dispersions were stored at 60° C. in a manner similar to Example 1. The changes of appearance after one-week storage were shown in Table 3. When the stabi-

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lizing agents were not used, coloration was observed. As opposed to that, the aqueous dispersions containing liposomes according to the present invention hardly exhibited change of appearance as compared with the dispersions before storage. Therefore, it was found that the advantage of the present invention, the stabilizing effect, can be obtained in the aqueous dispersions containing liposomes having various lipid composition containing yolk lecithin or soybean lecithin.

TABLE 3

Lipid composition ¹⁾ (Molar ratio)	Stabilizing agent ²⁾ (part by weight)	Appearance ³⁾
Present invention		
EPC/DMPC (1:0.1)	Sodium citrate + Sodium glutamate (0.5 + 0.06)	No change
EPC/DMPC (1:0.5)	Sodium citrate + Sodium glutamate (0.4 + 0.04)	No change
EPC/HEPC (1:0.1)	Sodium citrate + Sodium glutamate (0.5 + 0.06)	No change
EPC/DPPG (1:0.1)	Sodium citrate + Sodium glutamate (0.5 + 0.06)	No change
EPC/DPPA (1:0.1)	Sodium citrate + Sodium glutamate (0.5 + 0.06)	No change
EPC/Cho/DMPC(1:1:0.1)	Sodium citrate + Sodium glutamate (0.5 + 0.06)	No change
SPC/Cho/DMPC(1:1:0.1)	Sodium citrate + Sodium glutamate (0.5 + 0.06)	No change
Comparative examples		
EPC/DMPC (1:0.1)	—	****
EPC/DMPC (1:0.5)	—	****
EPC/HEPC (1:0.1)	—	****
EPC/DPPG (1:0.1)	—	****
EPC/DPPA (1:0.1)	—	****
EPC/Cho/DMPC(1:1:0.1)	—	****
SPC/Cho/DMPC(1:1:0.1)	—	****

¹⁾EPC: Egg phosphatidylcholine
SPC: Soybean phosphatidylcholine
DMPC: Dimyristoylphosphatidylcholine
HEPC: Hydrogenated egg phosphatidylcholine
DPPG: Dipalmitoylphosphatidylglycerol
DPPA: Dipalmitoylphosphatidic acid
Cho: Cholesterol
²⁾The amounts of the added stabilizing agents are shown in terms of parts by weight based on a part by weight of the lipids for forming liposomes. The amounts of the salts are represented by the amounts calculated as their free forms. (The concentrations of the lipids for forming liposomes were 27 to 36 mg/ml.)
³⁾Appearance was examined after one-week storage at 60° C.
****: Yellowish brown

Example 4

5 ml of Britton-Robinson buffer having a pH of 3 to 12 was added to the lipid film containing egg phosphatidylcholine prepared in Example 1 and strongly vortexed, and the obtained liquids were extruded under pressure through a polycarbonate membrane filter having a pore size of 0.2 μm (NUCLEOPORE®: available from Nomura Microscience Co., Ltd.) to give liposome stock dispersions having various pH values of 3 to 12.

To one part by volume of the above-obtained liposome stock dispersions having the pH values of 3 to 12, one part by volume portions of a solution containing 0.4 or 4% of sodium citrate and 0.4% of methionine and having the pH values of 3 to 12 were added to give aqueous dispersions containing liposomes having the pH value of 3 to 12 according to the present invention. For comparison, to one part by volume of the above liposome stock dispersions having the pH values of 3 to 12, one part by volume portions of the Britton-Robinson buffer of the same pH as the stock

dispersions were added to give comparative aqueous dispersions containing liposomes having the pH values of 3 to 12.

These dispersions were stored at 60° C. and their changes of appearance were examined in a manner similar to Example 1. The changes of appearance after one-week storage were shown in Table 4. While remarkable coloration was observed in the comparative examples, the aqueous dispersions containing liposomes according to the present invention, which were added with sodium citrate and methionine, exhibited no change in a pH range of 4 to 12 as compared with the dispersions before storage and hence they were stable. Therefore, it was found that the advantages of the present invention, the stabilizing effect, can be obtained in the pH range of 4 to 12.

TABLE 4

pH Stabilizing agent ¹⁾ (part by weight)	Appearance ²⁾
<u>3 Present invention</u>	
Sodium citrate + Methionine (0.05 + 0.08)	****
Sodium citrate + Methionine (0.5 + 0.08)	***
<u>Comparative example</u>	
—	****
<u>4 Present invention</u>	
Sodium citrate + Methionine (0.05 + 0.08)	No change
Sodium citrate + Methionine (0.5 + 0.08)	No change
<u>Comparative example</u>	
—	****
<u>5 Present invention</u>	
Sodium citrate + Methionine (0.05 + 0.08)	No change
Sodium citrate + Methionine (0.5 + 0.08)	No change
<u>Comparative example</u>	
—	****
<u>6 Present invention</u>	
Sodium citrate + Methionine (0.05 + 0.08)	No change
<u>Comparative Example</u>	
—	****
<u>7 Present invention</u>	
Sodium citrate + Methionine (0.05 + 0.08)	No change
Sodium citrate + Methionine (0.5 + 0.08)	No change
<u>Comparative Example</u>	
—	****
<u>8 Present invention</u>	
Sodium citrate + Methionine (0.05 + 0.08)	No change
<u>Comparative Example</u>	
—	****
<u>9 Present invention</u>	
Sodium citrate + Methionine (0.05 + 0.08)	No change
Sodium citrate + Methionine (0.5 + 0.08)	No change
<u>Comparative Example</u>	
—	****
<u>10 Present invention</u>	
Sodium citrate + Methionine (0.5 + 0.08)	No change
<u>Comparative Example</u>	
—	****
<u>11 Present invention</u>	
Sodium citrate + Methionine (0.5 + 0.08)	No change
<u>Comparative Example</u>	
—	****

TABLE 4-continued

pH Stabilizing agent ¹⁾ (part by weight)	Appearance ²⁾
<u>12 Present invention</u>	
Sodium citrate + Methionine (0.5 + 0.08)	No change
<u>Comparative Example</u>	
—	****

¹⁾The amounts of the added stabilizing agents are shown in terms of parts by weight based on a part by weight of the lipid for forming liposomes. The amounts of the salts are represented by the amounts calculated as their free forms. (The concentration of the lipid for forming liposomes was 25 mg/ml.)

²⁾Appearance was examined after one week storage at 60° C.

: Yellow, *: Yellowish brown

Example 5

5 ml of phosphate buffer⁷⁾ (made isotonic with sodium chloride) of pH 8.3 was added to the lipid film prepared in Example 1 containing egg phosphatidylcholine and strongly vortexed, and the obtained liquid was extruded under pressure through a polycarbonate membrane filter having a pore size of 0.2 μm (NUCLEOPORE®: available from Nommra Microscience Co., Ltd.) to give a milk white liposome stock dispersion.

One part by volume of a solution containing 0.4% of sodium citrate and 0.4% of methionine was added to one part by volume of the liposome stock dispersion obtained above to give an aqueous dispersion containing liposomes according to the present invention where the outside aqueous phases of the liposomes contained the stabilizing agents.

Moreover, 10 ml of phosphate buffer (made isotonic with sodium chloride) of pH 8.3 containing 0.2% of sodium citrate and 0.2% of methionine was added to the lipid film prepared in Example 1 containing egg phosphatidylcholine and strongly vortexed, and the obtained liquid was extruded under pressure through a polycarbonate membrane filter having a pore size of 0.2 μm (NUCLEOPORE®: available from Nommra Microscience Co., Ltd.) to give an aqueous dispersion containing liposomes of the present invention where the inside and outside aqueous phases of the liposomes contained the stabilizing agents.

For comparison, one part of phosphate buffer containing no stabilizing agents was added to one part of the above liposome stock dispersion to give a comparative aqueous dispersion containing liposomes. Moreover, the above-obtained aqueous dispersion containing liposomes according to the present invention where the inside and outside aqueous phases of the liposomes contained the stabilizing agents was centrifuged at 100,000g and the precipitated liposomes were dispersed again in the phosphate buffer to give a comparative aqueous dispersion containing liposomes where only the inside aqueous phases of the liposomes contained the stabilizing agents.

These dispersions were stored at 60° C. and the change of appearance was examined in a manner similar to Example 1. The changes of appearance after 2-week storage were shown in Table 5. When the stabilizing agents were not used and when they were added only to the inside aqueous phases of the liposomes, coloration was observed. On the other hand, the stabilizing agents were added to the inside and outside aqueous phases of the liposomes or only to the outside aqueous phase of liposomes, change was not observed as compared with the dispersions before storage. Therefore, it was found that the effect of the present invention is obtained

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by adding the stabilizing agents to the inside and outside aqueous phases of liposomes or to the outside aqueous phase of the liposomes.

TABLE 5

Stabilizing agent ¹⁾ (part by weight)	Appearance ²⁾
Present invention	
Sodium citrate + Methionine (0.05 + 0.05) (outside aqueous phase of liposomes)	No change
Sodium citrate + Methionine (0.05 + 0.05) (outside and inside aqueous phases of liposomes)	No change
Comparative examples	
Sodium citrate + Methionine (0.05 + 0.05) (inside aqueous phase of liposomes)	***
—	****

¹⁾The amounts of the added stabilizing agents are shown in terms of parts by weight based on a part by weight of the lipid for forming liposomes. The amounts of the salts are represented by the amounts calculated as their free forms. (The concentration of the lipid for forming liposomes was 25 mg/ml.)
²⁾Appearance was examined after 2-week storage at 60° C.
: Yellow, *: Yellowish brown

Example 6

5 ml of phosphate buffer (made isotonic with sodium chloride) of pH 8.3 containing 0.4% of sodium betamethasone phosphate was added to the lipid film prepared in Example 1 containing egg phosphatidylcholine and strongly vortexed. This aqueous dispersion containing liposomes was frozen with dry ice/acetone and thawed in a water bath at 20° C. After repeating this freeze-thawing process eight times, the dispersion was extruded under pressure through a polycarbonate membrane filter having a pore size of 0.2 μm (NUCLEOPORE ®: available from Nomura Microscience Co., Ltd.). Further, this aqueous dispersion containing liposomes was centrifuged at 100,000g to remove unencapsulated drug and was dispersing again the precipitated liposomes in phosphate buffer to give a liposome stock dispersion containing sodium betamethasone phosphate.

0.25 parts by volume of phosphate buffer containing 1.6% of sodium citrate and 0.8% of sodium aspartate or one part by volume of phosphate buffer containing 0.4% sodium citrate and 0.2% of sodium aspartate was added to one part by volume of the liposome stock dispersion obtained above to give aqueous dispersions containing liposomes according to the present invention having concentrations of the lipid for forming liposomes of 0.04 and 0.025 parts by weight (based on a part by weight of the buffer where the lipid was dispersed). Moreover, the above liposome stock dispersion was diluted with phosphate buffer by five times or fifty times, and one part by volume of phosphate buffer containing 0.04% of sodium citrate and 0.02% of sodium aspartate or one part by volume of phosphate buffer containing 0.004% of sodium citrate and 0.002% of sodium aspartate was added to one part by volume of the diluted dispersions to give aqueous dispersions containing liposomes according to the present invention having a concentration of the lipid for forming liposomes of 0.0025 or 0.00025 parts by weight. For comparison, a dispersion was prepared in a manner similar to the above except that sodium citrate and sodium aspartate were not added.

These dispersions were stored at 60° C. in a manner similar to Example 1. Changes of appearance and relative encapsulation efficiencies after 2-week storage are shown in Table 6. When the stabilizing agents were not contained, change of appearance was observed and the relative encapsulation efficiency was remarkably decreased at a concentration of the lipid for forming liposomes of not less than 0.0025 parts by weight. When the concentration of the lipid for forming liposomes was 0.00025 parts by weight, though change of appearance was not observed at this concentration, the decrease of the relative encapsulation efficiency was remarkable. On the other hand, the aqueous dispersion containing liposomes of the present invention hardly exhibited change of appearance and the relative encapsulation efficiency was more than about 90% at all of the concentrations of the lipid for forming liposomes. Therefore, it was found that the aqueous dispersions containing liposomes of the present invention were stable regardless of the concentration of the lipid for forming liposomes.

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ulation efficiency was remarkably decreased at a concentration of the lipid for forming liposomes of not less than 0.0025 parts by weight. When the concentration of the lipid for forming liposomes was 0.00025 parts by weight, though change of appearance was not observed at this concentration, the decrease of the relative encapsulation efficiency was remarkable. On the other hand, the aqueous dispersion containing liposomes of the present invention hardly exhibited change of appearance and the relative encapsulation efficiency was more than about 90% at all of the concentrations of the lipid for forming liposomes. Therefore, it was found that the aqueous dispersions containing liposomes of the present invention were stable regardless of the concentration of the lipid for forming liposomes.

TABLE 6

Concentration of lipid for forming liposomes ¹⁾	Stabilizing agent ²⁾	Appearance ³⁾	Relative encapsulation efficiency (%)
<u>Present invention</u>			
0.04	Sodium citrate + Sodium aspartate (0.05 + 0.05)	*	89.5
0.025		*	90.9
0.0025		No change	100.0
0.00025		No change	100.0
<u>Comparative examples</u>			
0.04	Not present	****	65.8
0.025		****	49.0
0.0025		**	11.5
0.00025		No change	2.8

¹⁾The concentrations of the lipid for forming liposomes are indicated in terms of parts by weight based on a part by weight of the buffer where the lipid was dispersed.
²⁾The amounts of the added stabilizing agents are shown in terms of parts by weight based on a part by weight of the lipid for forming liposomes. The amounts of the salts are represented by the amounts calculated as their free forms.
³⁾Appearance was examined after 2-week storage at 60° C.
*: Faint yellow, **: Light yellow, ****: Yellowish brown

Example 7

To one part by volume of the liposome stock dispersion prepared in Example 6 containing sodium betamethasone phosphate, one part by volume of a solution containing sodium citrate and an amino acid selected from various amino acids was added to give aqueous dispersions containing liposomes of the present invention. For comparison, to one part by volume of the above liposome stock dispersion, one part by volume of phosphate buffer containing sodium citrate or an amino acid alone or phosphate buffer containing no stabilizing agents was added to give comparative aqueous dispersions containing liposomes.

These dispersions were stored at 60° C. in a manner similar to Example 1. Changes of appearance after one-week storage are shown in Table 7. When the stabilizing agents were not contained and when a hydroxy acid or an amino acid alone was added, coloration was observed. On the other hand, the aqueous dispersions containing liposomes of the present invention hardly exhibited change of appearance as compared with the dispersions before storage. Therefore, it was found that the aqueous dispersions containing liposomes of the present invention were stable even if they contained a drug.

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TABLE 7

Liposomes encapsulating sodium betamethasone phosphate		
Stabilizing agent ¹⁾ (part by weight)		
Hydroxy acid	Amino acid	Appearance ²⁾
Present invention		
Sodium citrate (0.05)	Glycine (0.08)	No change
Sodium citrate (0.05)	Alanine (0.08)	No change
Sodium citrate (0.05)	Leucine (0.08)	No change
Sodium citrate (0.05)	Serine (0.08)	No change
Sodium citrate (0.05)	Proline (0.08)	No change
Sodium citrate (0.05)	Hydroxyproline (0.08)	No change
Sodium citrate (0.05)	Cysteine (0.08)	No change
Sodium citrate (0.05)	Lysine (0.08)	No change
Sodium citrate (0.05)	Sodium glutamate (0.06)	No change
Sodium citrate (0.05)	Sodium aspartate (0.06)	No change
Comparative examples		
—	—	***
Sodium citrate (0.05)	—	*
—	Glycine (0.08)	**
—	Alanine (0.08)	**
—	Leucine (0.08)	*
—	Serine (0.08)	**
—	Proline (0.08)	*
—	Hydroxyproline (0.08)	**
—	Cysteine (0.08)	**
—	Lysine (0.08)	***
—	Sodium glutamate (0.06)	*
—	Sodium aspartate (0.06)	*

¹⁾The amounts of the added stabilizing agents are shown in terms of parts by weight based on a part by weight of the lipid for forming liposomes. The amounts of the salts are represented by the amounts calculated as their free forms. (The concentration of the lipid for forming liposomes was 25 mg/ml.)
²⁾Appearance was examined after one-week storage at 60° C.
*: Faint yellow, **: Light yellow, ***: Yellow, ****: Yellowish brown

Example 8

(Liposomes encapsulating sodium betamethasone phosphate)

To one part by volume of the liposome stock dispersion prepared in Example 6 containing sodium betamethasone phosphate, one part by volume of a solution containing 4% of sodium citrate and 0.4 % of sodium glutamate was added to give an aqueous dispersion containing liposomes of the present invention. For comparison, a dispersion was prepared in a manner similar to the above except that sodium glutamate and sodium citrate were not added and nitrogen gas substitution was carried out when it was filled into ampoules.

These dispersions were stored at 60° C. to examine changes of appearance in a manner similar to Example 1. Changes of appearance, relative encapsulation efficiencies and drug-remaining ratios after one or two-week storage are shown in Table 8. The comparative aqueous dispersion containing liposomes exhibited change of color with the lapse of time in spite of the nitrogen-substitution and, simultaneously, the encapsulation efficiency was decreased. On the other hand, the aqueous dispersion containing liposomes of the present invention exhibited no change of appearance even after two-week storage at 60° C. and maintained about 90% of the relative encapsulation efficiency and the drug-remaining ratio.

TABLE 8

<u>Liposomes encapsulating sodium betamethasone phosphate</u>		
	Present invention Stabilizing agent Sodium citrate + Sodium glutamate (0.5 + 0.06)	Comparative example No stabilizing agent
After 1-week storage at 60° C.	Appearance ¹⁾ Relative encapsulation efficiency (%) Drug remaining ratio (%)	** 82.4 91.5
After 2-week storage at 60° C.	Appearance ¹⁾ Relative encapsulation efficiency (%) Drug-remaining ratio (%)	*** 51.4 57.4

¹⁾The amounts of the added stabilizing agents are shown in terms of parts by weight based on a part by weight of the lipid for forming liposomes. The amounts of the salts are represented by the amounts calculated as their free forms. (The concentration of the lipid for forming liposomes was 25 mg/ml.)
*: Light yellow, **: Yellow, ***: Yellowish brown

Example 9

(Liposomes encapsulating morphine hydrochloride)

In a manner similar to Example 6 except that 5 ml of phosphate buffer of pH 6.0 (made isotonic with sodium chloride) containing 0.5% of morphine hydrochloride was used instead of 5 ml of phosphate buffer of pH 8.3 containing 0.4% of sodium betamethasone phosphate in Example 6, a liposome stock dispersion was obtained.

To one part by volume of the liposome stock dispersion obtained above, one part by volume of phosphate buffer containing 0.4% of sodium citrate and 0.4% of sodium glutamate was added to give an aqueous dispersion containing liposomes of the present invention. For comparison, to one part by volume of the above liposome stock dispersion of which liposomes encapsulated morphine, one part by volume of phosphate buffer containing 0.8% of sodium citrate or 0.8% of sodium glutamate alone or one part by volume of phosphate buffer containing no stabilizing agents was added to give comparative aqueous dispersions containing liposomes.

These dispersions were stored at 60° C. in a manner similar to Example 1. Changes of appearance, relative encapsulation efficiencies and drug-remaining ratios after 2-week storage are shown in Table 9. When the stabilizing agents were not used, changes of appearance and remarkable decreases of the encapsulation efficiency and the drug-remaining ratio were observed. Moreover, when sodium citrate or sodium glutamate was added alone, though change of appearance and decrease of the drug-remaining ratio were slight, decrease of the relative encapsulation efficiency was remarkable. On the other hand, the aqueous dispersion containing liposomes of the present invention showed no change of appearance and no decrease of the drug-remaining ratio and maintained about 90% of the relative encapsulation efficiency.

From the results of Examples 8 and 9, it was found that the aqueous dispersion containing liposomes of the present invention can prevent change of appearance and, moreover, can prevent the leak of drugs.

TABLE 9

Liposomes encapsulating morphine hydrochloride			
Stabilizing agent ¹⁾ (part by weight)	Appearance ²⁾	Relative encapsulation efficiency (%)	Drug- retaining ratio (%)
Present invention			
Sodium citrate + Sodium glutamate (0.05 + 0.08)	No change	90.7	98.9
Comparative examples			
Sodium citrate (0.1)	*	80.5	95.7
Sodium glutamate (0.12)	*	79.3	97.5
Not present	****	59.8	75.9

¹⁾The amounts of the added stabilizing agents are shown in terms of parts by weight based on a part by weight of lipid for forming liposomes. The amounts of the salts are represented by the amounts calculated as their free forms. (The concentration of the lipid for forming liposomes was 25 mg/ml.)
²⁾Appearance was examined after 2-week storage at 60° C.
*: Faint yellow, ****: Yellowish brown

Example 10

(Liposomes encapsulating sodium dexamethasone phosphate)

In a manner similar to Example 6 except that 5 ml of phosphate buffer of pH 8.3 containing 0.4% of sodium betamethasone phosphate in Example 6 was replaced with 5 ml of phosphate buffer of pH 8.3 containing 0.4% of sodium dexamethasone phosphate, a liposome stock dispersion containing sodium dexamethasone phosphate was obtained.

Example 11

(Liposomes encapsulating timolol maleate)

In a manner similar to Example 6 except that 5 ml of phosphate buffer of pH 8.3 containing 0.4% of sodium betamethasone phosphate in Example 6 was replaced with 5 ml of phosphate buffer of pH 8.0 containing 5% of timolol maleate, a liposome stock dispersion containing timolol maleate was obtained.

Example 12

(Liposomes encapsulating sodium diclofenac)

5 ml of phosphate buffer (made isotonic with sodium chloride) of pH 8.0 containing 0.2% of sodium diclofenac was added to the lipid film prepared in Example 1 containing egg phosphatidylcholine and strongly vortexed. The obtained aqueous dispersion containing liposomes was extruded under pressure through a polycarbonate membrane filter having a pore size of 0.2 μm (NUCLEOPORE®: available from Nomura Microscience Co., Ltd.) to give a liposome stock dispersion containing sodium diclofenac.

Example 13

(Liposomes encapsulating fluorometholone)

0.25 g of egg phosphatidylcholine, 0.125 g of cholesterol and 1 mg of fluorometholone were dissolved in 40 ml of a hexane/methanol mixture (10:1), and the solvent was removed by an evaporator to form a lipid film containing fluorometholone. 5 ml of phosphate buffer (made isotonic with sodium chloride) of pH 8.0 was added to the lipid film and strongly vortexed, and the obtained aqueous dispersion containing liposomes was extruded under pressure through

a polycarbonate membrane filter having a pore size of 0.2 μm (NUCLEOPORE®: available from Nomura Microscience Co., Ltd.) to give a liposome stock dispersion containing fluorometholone.

Example 14

(Liposomes encapsulating sodium pranoprofen)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of phosphate buffer of pH 7.0 containing 0.1% of sodium pranoprofen, a liposome stock dispersion containing sodium pranoprofen was obtained.

Example 15

(Liposomes encapsulating tegafur)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 9.0 containing 4% of tegafur, a liposome stock dispersion containing tegafur was obtained.

Example 16

(Liposomes encapsulating gentamicin sulfate)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 6.0 containing 0.3% of gentamicin sulfate, a liposome stock dispersion containing gentamicin sulfate was obtained.

Example 17

(Liposomes encapsulating chlorpheniramine maleate)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 5.0 containing 0.5% of chlorpheniramine maleate, a liposome stock dispersion containing chlorpheniramine maleate was obtained.

Example 18

(Liposomes encapsulating sodium cromoglicate)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 5.5 containing 2% of sodium cromoglicate, a liposome stock dispersion containing sodium cromoglicate was obtained.

Example 19

(Liposomes encapsulating urokinase)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 7.0 containing 12,000 units/ml of urokinase, a liposome stock dispersion containing urokinase was obtained.

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Example 20

(Liposomes encapsulating lidocaine hydrochloride)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 6.0 containing 1% of lidocaine hydrochloride, a liposome stock dispersion containing lidocaine hydrochloride was obtained.

Example 21

(Liposomes encapsulating phenylephrine hydrochloride)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 5.0 containing 0.5% of phenylephrine hydrochloride, a liposome stock dispersion containing phenylephrine hydrochloride was obtained.

Example 22

(Liposomes encapsulating pilocarpine hydrochloride)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 5.0 containing 1% of pilocarpine hydrochloride, a liposome stock dispersion containing pilocarpine hydrochloride was obtained.

Example 23

(Liposomes encapsulating carbachol)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 7.0 containing 0.75% of carbachol, a liposome stock dispersion containing carbachol was obtained.

Example 24

(Liposomes encapsulating TAS)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 5.0 containing 0.2% of TAS, a liposome stock dispersion containing TAS was obtained.

Example 25

(Liposomes encapsulating prostaglandin E₁)

In a manner similar to Example 13 except that 1 mg of fluorometholon was replaced with 25 µg of prostaglandin E₁ and phosphate buffer of pH 8.0 was replaced with Britton-Robinson buffer of pH 5.0, a liposome stock dispersion containing prostaglandin E₁ was obtained.

Example 26

(Liposomes encapsulating tropicamide)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 5.0 containing 0.5% of

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tropicamide, a liposome stock dispersion containing tropicamide was obtained.

Example 27

(Liposomes encapsulating glutathione)

In a manner similar to Example 12 except that 5 ml of phosphate buffer of pH 8.0 containing 0.2% of sodium diclofenac in Example 12 was replaced with 5 ml of Britton-Robinson buffer of pH 6.0 containing 2% of glutathione, a liposome stock dispersion containing glutathione was obtained.

Example 28

(Liposomes encapsulating penicillamine)

To the lipid film prepared in Example 1 containing egg phosphatidylcholine, 5 ml of Britton-Robinson buffer of pH 5.0 containing 1% of penicillamine was added and strongly vortexed. The obtained aqueous dispersion containing liposomes was extruded under pressure through a polycarbonate membrane filter having a pore size of 0.2 µm (NUCLEOPORE®; available from Nommura Microscience Co., Ltd.) and centrifuged at 100,000×g to remove unencapsulated drug and was dispersing again the precipitated liposomes in Britton-Robinson buffer to give a liposome stock dispersion containing penicillamine.

To one part by volume of each of the liposome stock dispersions obtained in Examples 10 to 28 containing various drugs, one part by volume of Britton-Robinson buffer containing sodium citrate and sodium glutamate, sodium aspartate or histidine hydrochloride at a predetermined concentration was added to give aqueous dispersions containing liposomes of the present invention shown in Table 10. For comparison, to one part by volume of the above liposome stock dispersions, Britton-Robinson buffer containing sodium citrate or one of the aforementioned amino acids alone or Britton-Robinson buffer containing no stabilizing agents was added to give comparative aqueous dispersions containing liposomes.

These dispersions were stored at 60° C. in a manner similar to Example 1. Changes of appearance after one or two-week storage were shown in Table 10. Coloration was observed when the stabilizing agents were not used or when sodium citrate or an amino acid alone was added. As opposed to that, the aqueous dispersions containing liposomes of the present invention hardly exhibited change of appearance as compared with the dispersions before storage. From the above results, it was found that the aqueous dispersions containing liposomes of the present invention were stable whether the encapsulated drug was hydrophilic or lipophilic.

TABLE 10

Ex- ample	Stabilizing agent ¹⁾ (part by weight)	Appearance ²⁾
10	Present invention	
	Sodium citrate + Sodium glutamate (0.5 + 0.05)	No change
	Comparative examples	
	Sodium citrate (1.0)	++

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TABLE 10-continued

Ex- ample	Stabilizing agent ¹⁾ (part by weight)	Appearance ²⁾	
11	Sodium glutamate (0.12)	***	5
	Not present	****	
	Present invention		
12	Sodium citrate + Sodium glutamate (0.125 + 0.15)	No change	10
	Comparative examples		
	Sodium citrate (0.25) Sodium glutamate (0.3) Not present	.. . ****	
13	Sodium citrate + Sodium glutamate (0.125 + 0.06)	No change	15
	Comparative examples		
	Sodium citrate (0.25) Sodium glutamate (0.12) Not present	.. **** ****	
14	Sodium citrate + Sodium aspartate (0.05 + 0.06)	No change	20
	Comparative examples		
	Sodium citrate (0.1) Sodium aspartate (0.12) Not present	 ****	
15	Sodium citrate + Histidine hydrochloride (0.125 + 0.013)	No change	25
	Comparative examples		
	Sodium citrate (0.25) Histidine hydrochloride (0.03) Not present	 ****	
16	Sodium citrate + Histidine hydrochloride (0.25 + 0.006)	No change	30
	Comparative examples		
	Sodium citrate (0.5) Histidine hydrochloride (0.012) Not present	**** **** ****	
17	Sodium citrate + Histidine hydrochloride (0.25 + 0.006)	No change	35
	Comparative examples		
	Sodium citrate (0.5) Histidine hydrochloride (0.012) Not present	.. **** ****	
18	Sodium citrate + Histidine hydrochloride (0.25 + 0.006)	No change	40
	Comparative examples		
	Sodium citrate (0.5) Histidine hydrochloride (0.012) Not present	 ****	
19	Sodium citrate + Histidine hydrochloride (0.25 + 0.006)	No change	45
	Comparative examples		
	Sodium citrate (0.5) Histidine hydrochloride (0.012) Not present	.. **** ****	
20	Sodium citrate + Sodium aspartate (0.25 + 0.06)	No change	50
	Comparative examples		
	Sodium citrate (0.5) Sodium aspartate (0.12) Not present	.. **** ****	
21	Sodium citrate + Sodium aspartate (0.25 + 0.06)	No change	55
	Comparative examples		
	Sodium citrate (0.5) Sodium aspartate (0.12) Not present	.. **** ****	
22	Sodium citrate + Sodium aspartate (0.25 + 0.06)	No change	60
	Comparative examples		
	Sodium citrate (0.5) Sodium aspartate (0.12) Not present	.. **** ****	

TABLE 10-continued

Ex- ample	Stabilizing agent ¹⁾ (part by weight)	Appearance ²⁾
19	Not present	****
	Present invention	
	Sodium citrate + Histidine hydrochloride (0.25 + 0.006)	.
20	Sodium citrate (0.5) Histidine hydrochloride (0.012) Not present	
	Present invention	
	Sodium citrate + Sodium aspartate (0.25 + 0.06)	No change
21	Sodium citrate (0.5) Sodium aspartate (0.12) Not present	 ****
	Present invention	
	Sodium citrate + Sodium aspartate (0.25 + 0.06)	No change
22	Sodium citrate (0.5) Sodium aspartate (0.12) Not present	. **** ****
	Present invention	
	Sodium citrate + Sodium aspartate (0.25 + 0.06)	No change
23	Sodium citrate (0.5) Sodium aspartate (0.12) Not present	.. **** ****
	Present invention	
	Sodium citrate + Sodium aspartate (0.25 + 0.06)	No change
24	Sodium citrate (0.5) Sodium Aspartate (0.12) Not present	. .. ****
	Present invention	
	Sodium citrate + Sodium aspartate (0.25 + 0.06)	.
25	Sodium citrate (0.5) Sodium aspartate (0.12) Not present	 ****
	Present invention	
	Sodium citrate + Histidine hydrochloride (0.25 + 0.006)	No change
26	Sodium citrate (0.5) Histidine hydrochloride (0.012) Not present	 ****
	Present invention	
	Sodium citrate + Sodium aspartate (0.25 + 0.06)	.
27	Sodium citrate (0.5) Sodium aspartate (0.12) Not present	.. **** ****
	Present invention	
	Sodium citrate + Sodium aspartate (0.25 + 0.06)	No change

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TABLE 10-continued

Ex- ample	Stabilizing agent ¹⁾ (part by weight)	Appearance ²⁾
27	<u>Present invention</u>	
	Sodium citrate + Sodium aspartate (0.25 + 0.06)	No change
	<u>Comparative examples</u>	
	Sodium citrate (0.5)	**
	Sodium aspartate (0.12)	***
	Not present	****
28	<u>Present invention</u>	
	Sodium citrate + Histidine hydrochloride (0.25 + 0.06)	No change
	<u>Comparative examples</u>	
	Sodium citrate (0.5)	*
	Histidine hydrochloride (0.012)	**
	Not present	**

¹⁾The amounts of the added stabilizing agents are shown in terms of parts by weight based on a part by weight of the lipid for forming liposomes. The amounts of the salts are represented by the amounts calculated as their free forms. (The concentration of liposome membrane forming materials was 25 g/gel.)

²⁾Appearance was examined after 2-week storage for Examples 10 to 25 and after one-week storage for Examples 26 to 28.

*: Faint yellow, **: Light yellow, ***: Yellow, ****: Yellowish brown

Example 29

(Parenteral injection)

The aqueous dispersion containing liposomes obtained in Example 8 containing 2% of sodium citrate and 0.2% of sodium glutamate, of which liposomes encapsulated betamethasone phosphate, was diluted with phosphate buffer (pH 8.3) containing 2% of sodium citrate and 0.2% of sodium glutamate to give an aqueous dispersion containing liposomes, which contained 1 mg of betamethasone per 1 ml. The dispersion was filled into 2 ml of glass ampoules and sterilized by heating at 70° C. for 1 hour three times every 24 hours to give a parenteral injection.

Example 30

(Nasal drop)

The aqueous dispersion containing liposomes obtained in Example 8 containing 2% of sodium citrate and 0.2% of sodium glutamate, of which liposomes encapsulated betamethasone phosphate, was diluted with phosphate buffer (pH 8.3) containing 2% of sodium citrate and 0.2% of sodium glutamate and, further, added with benzalkonium chloride of which final concentration in the dispersion was 0.005% to give an aqueous dispersion containing 1 mg of betamethasone per 1 ml. This dispersion was filtered through a membrane filter of 0.22 µm and filled in a container for spraying as a nasal drop.

Example 31

(Endermic agent)

The aqueous dispersion containing liposomes obtained in Example 8 containing 2% of sodium citrate and 0.2% of sodium glutamate, of which liposomes encapsulated betamethasone phosphate, was diluted with phosphate buffer (pH 8.3) containing 2% of sodium citrate and 0.2% of sodium glutamate and, further, added with benzalkonium chloride of which final concentration in the dispersion was 0.005% to give an aqueous dispersion containing 1 mg of

betamethasone per 1 ml. This dispersion was filtered through a membrane filter of 0.22 µm and filled in a plastic container with sponge as an endermic agent.

Example 32

(Inhalation agent)

The aqueous dispersion containing liposomes obtained in Example 8 containing 2% of sodium citrate and 0.2% of sodium glutamate, of which liposomes encapsulated betamethasone phosphate, was diluted with phosphate buffer (pH 8.3) containing 2% of sodium citrate and 0.2% of sodium glutamate and, further, added with benzalkonium chloride of which final concentration in the dispersion was 0.005% to give an aqueous dispersion containing 1 mg of betamethasone per 1 ml. This dispersion was filtered through a membrane filter of 0.22 µm and filled in a nebulizer container as an inhalation agent.

Example 33

(Oral agent)

The aqueous dispersion containing liposomes obtained in Example 8 containing 2% of sodium citrate and 0.2% of sodium glutamate, of which liposomes encapsulated betamethasone phosphate, was diluted with phosphate buffer (pH 8.3) containing 2% of sodium citrate and 0.2% of sodium glutamate and, further, added with D-sorbitol and potassium sorbate of which final concentrations in the dispersion were 10% and 0.1% respectively to give an aqueous dispersion containing 1 mg of betamethasone per 1 ml. This dispersion was filtered through a membrane filter of 0.45 µm and filled in a glass container as an oral agent.

Example 34

(Parenteral injection)

The aqueous dispersion containing liposomes obtained in Example 9 containing 0.2% of sodium citrate and 0.2% of sodium glutamate, of which liposomes encapsulated morphine hydrochloride, was diluted with phosphate buffer (pH 6.0) containing 0.2% of sodium citrate and 0.2% of sodium glutamate to give an aqueous dispersion containing 0.5 mg of morphine hydrochloride per 1 ml. This dispersion was filtered through a membrane filter of 0.22 µm and filled in a glass ampoule as a parenteral injection.

Example 35

(Eye drop)

The aqueous dispersion containing liposomes obtained in Example 11 containing 0.5% of sodium citrate and 0.5% of sodium glutamate, of which liposomes encapsulated timolol maleate, was diluted with phosphate buffer (pH 8.0) containing 0.5% of sodium citrate and 0.5% of sodium glutamate and, further, added with benzalkonium chloride of which final concentration in the dispersion was 0.005% to give an aqueous dispersion containing 2.5 mg of timolol per 1 ml. This dispersion was filtered through a membrane filter of 0.22 µm and filled in a 5-ml eye drop bottle as an eye drop.

Example 36

(Eye drop)

The aqueous dispersion containing obtained in Example 12 containing 0.5% of sodium citrate and 0.2% of sodium glutamate, of which liposomes encapsulated sodium diclofenac, was diluted with phosphate buffer (pH 8.0) containing

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0.5% of sodium citrate and 0.2% of sodium glutamate and, further, added with benzalkonium chloride of which final concentration in the dispersion was 0.005% to give an aqueous dispersion containing 1 mg of sodium diclofenac per 1 ml. This dispersion was filtered through a membrane filter of 0.22 μ m and filled in a 5-ml eye drop bottle as an eye drop.

According to the present invention, there are provided aqueous dispersions containing liposomes which hardly show coloration though they are composed of natural phospholipids, which are inexpensive and safe, show little leak of drugs encapsulated therein and hence are excellent in storage stability.

Moreover, since the advantages of the present invention are obtained in a broad pH range, it is possible to select a pH value suitable for stably storing the encapsulated drug and, therefore, there are provided aqueous dispersion containing liposomes wherein the storage stability of the encapsulated drug is also excellent.

What is claimed is:

1. An aqueous dispersion containing liposomes comprising one or both of yolk lecithin and soybean lecithin as lipids for forming liposomes wherein the dispersion contains citric acid or a pharmaceutically acceptable salt thereof and one or more amino acids selected from the group consisting of methionine, histidine, arginine, glutamic acid, aspartic acid and pharmaceutically acceptable salts thereof, wherein said citric acid and amino acids or pharmaceutically acceptable salts thereof, if said particular amino acid or pharmaceutically acceptable salt thereof is present, are contained in the following parts by weight ranges wherein the part by weight of the amino acid salt refers to the amount by weight expressed in terms of the particular amino acid:

citric acid or a pharmaceutically acceptable salt is present in an amount ranging from 0.05 to 5 parts by weight based on a part by weight of the lipids used to form the liposomes and not more than 0.13 parts by weight of the aqueous dispersion containing liposomes in terms of citric acid;

histidine or pharmaceutically acceptable salts thereof, if present, is or are contained in an amount expressed in terms of histidine ranging from 0.0006 to 0.3 parts by weight based on one part by weight of the lipids used to form the liposomes and not more than 0.015 parts by weight based on one part by weight of the aqueous dispersion containing liposomes;

arginine or pharmaceutically acceptable salts thereof, if present, is or are contained in an amount expressed in terms of arginine, ranging from 0.02 to 4 parts by weight based on one part by weight of the lipids used to form liposomes and not more than 0.05 parts by weight based on one part by weight of the aqueous dispersion containing liposomes;

methionine or pharmaceutically acceptable salts thereof, if present, is or are contained in an amount ranging from 0.008 to 0.8 parts by weight based on one part by weight of the lipids and to make the liposomes and not more than 0.02 parts by weight based on one part by weight of the aqueous dispersion containing liposomes; and

wherein glutamic acid and aspartic acid or pharmaceutically acceptable salts thereof, if present, are contained

in an amount by weight expressed in terms of glutamic acid and aspartic acid ranging from 0.015 to 3 parts by weight based on one part by weight of the lipids used to form the liposomes and not more than 0.08 parts by weight based on one part by weight of the aqueous dispersion containing liposomes; and

wherein the amount of citric acid and the one or more amino acids or pharmaceutically acceptable salts thereof are sufficient to produce an aqueous dispersion containing liposomes which does not substantially change color during storage.

2. The aqueous dispersion containing dispersion of claim 1 wherein color change is evaluated using a color difference meter before and after storage using coordinate values based on the UCS system of color representation, L , a and b are measured based upon the color difference of the aqueous dispersion before and after storage, and wherein relative color change, ΔE , is calculated according to the equation:

$$\Delta E = \{(L_1 - L_2)^2 + (a_1 - a_2)^2 + (b_1 - b_2)^2\}^{1/2}$$

and wherein an aqueous liposome composition which does not substantially change color during storage refers to a composition which possesses a ΔE of 3.5 or less.

3. The aqueous dispersion of claim 1 wherein the amount of citric acid is about 0.05 parts by weight and the amount of the amino acid or a pharmaceutically acceptable salt thereof ranges from about 0.06 to about 0.08 parts by weight.

4. The aqueous dispersion containing liposomes according to claim 1 wherein the liposomes encapsulate a drug.

5. The aqueous dispersion containing liposomes according to claim 4 wherein the drug is any one selected from the group consisting of hypnotics, sedatives, anti-anxiety agents, antiepileptics, antipyretics, analgesics, anti-inflammatory agents, psychotropic agents, local anesthetics, antispasmodics, antihypertensives, vasoconstrictors, vasodilators, antihyperlipemia agents, antitussives, bronchodilators, anticancer agents, hormones, vitamins, hemostatics, enzymes, agents suitable for treatment of diabetes, carcinostatic agents, anti-allergic agents, antibiotics, chemotherapeutics, antiglaucoma drugs, anticataract drugs, mydriatics, miotics, diagnostic agents, prostaglandins and immunoregulatory drugs.

6. The aqueous dispersion containing liposomes according to claim 5 wherein the drug is any one selected from the group consisting of tegafur, gentamicin, penicillamine, chlorpheniramine, cromoglicic acid, betamethasone phosphate, dexamethasone phosphate, diclofenac, fluorometholone, pranoprofen, morphine, urokinase, lidocaine, prostaglandin E_1 , 2-N-[3-(1-piperidinomethyl)phenoxy]propyl]amino-5-amino-1,3,4-thiadiazole, timolol, glutathione, phenylephrine, tropicamide, pilocarpine, carbachol epikratex and pharmaceutically acceptable salts thereof.

7. A pharmaceutical preparation which is in the form of a composition administrable by parenteral injection, a nasal drop, a lotion, a liniment, a composition administrable by inhalation, or a composition suitable for oral administration which comprises the aqueous dispersion containing liposomes according to claim 4.

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/22 // (A61K 38/22, 47:10, 47:12) (A61K 38/22, 47:12, 47:26)	A1	(11) International Publication Number: WO 98/11912 (43) International Publication Date: 26 March 1998 (26.03.98)
(21) International Application Number: PCT/US97/16420 (22) International Filing Date: 17 September 1997 (17.09.97) (30) Priority Data: 08/717,306 20 September 1996 (20.09.96) US (71) Applicant: NEUROBIOLOGICAL TECHNOLOGIES, INC. [US/US]; 1387 Marina Way South, Richmond, CA 94804 (US). (72) Inventors: HO, Bert; 101 Bacon Court, Lafayette, CA 94549 (US). KHOSROVI, Behzad; 8351 Kent Court, El Cerrito, CA 94530 (US). HIRTZER, Pamela, G.; 291 Scond Avenue, Piedmont, CA 94611 (US). (74) Agents: CHICKERING, Robert, B. et al.; Flehr, Hohbach, Test, Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).		(81) Designated States: AU, CA, JP, NO, RU, UA, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: PHARMACEUTICAL FORMULATIONS OF CORTICOTROPIN RELEASING FACTOR HAVING IMPROVED STABIL- ITY IN LIQUID FORM (57) Abstract The present invention relates to novel formulations of corticotropin releasing factor (CRF) having improved stability during storage over extended periods of time as liquids, which retain significant pharmaceutical activity during such storage and/or which are physiologically compatible. The formulations of the present invention comprise: (a) a pharmaceutically effective concentration of CRF; (b) a buffer to maintain the pH of the formulation between about 2.0 to about 5.0 or about 6.0 to about 9.0 when in liquid form and (c) an alcohol such as mannitol, sorbitol, methanol, glycerol, or the like. Other aspects of the present invention involve addition of various other components to the formulations described herein for the purpose of decreasing the rate of degradation of the CRF peptide or increasing its solubility in solution. Finally, the present invention is further directed to methods for preparing such novel pharmaceutical formulations of CRF and methods of using these formulations to reduce inflammation and edema in mammals.		

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The publications and other materials cited herein are used to illuminate the background of the invention and to provide additional details with regard to its practice. Each of the publications referenced herein are hereby expressly incorporated by reference in their entirety.

5

SUMMARY OF THE INVENTION

The present invention is predicated upon the identification and preparation of novel formulations of CRF having acceptable solubility and which are stable when stored as liquids for extended periods of time, which do not lose significant pharmaceutical activity during such storage and/or which are
10 pharmaceutically acceptable in that they are well tolerated in terms of irritation when administered. Thus, one aspect of the present invention relates to pharmaceutically acceptable formulations of CRF comprising (a) a pharmaceutically effective amount of CRF, (b) a buffer at a concentration of from about 1 mM to about 50 mM which functions to maintain the pH of said
15 formulation between about 2.0 to about 5.0 or between/about 6.0 to about 9.0 when in liquid form, wherein the anion of said buffer is selected from the group consisting of acetate, phosphate, carbonate, succinate, citrate, borate, tartrate, fumarate and lactate and (c) an alcohol, in a concentration of from about 1% to about 10%, wherein said alcohol is selected from the group
20 consisting of mannitol, sorbitol, ribitol, arabitol, xylitol, inositol, galactitol, methanol, ethanol and glycerol.

In other aspects of the present invention, various components are optionally included in the formulation as described above and may replace the buffer. Such additives include amino acids such as methionine, arginine, lysine,
25 glutamic acid, cysteine, glutathione, and the like. Amino acids are generally present in concentrations ranging from about 1 mM to about 100 mM.

In yet other aspects of the present invention, various sugars are optionally included in the CRF formulations as described above. Such sugars include, for example, glucose, sucrose, lactose, fructose, trehalose, mannose, and

The following examples are provided merely to illustrate various embodiments of the present invention and are not intended to be limiting of the subject matter disclosed herein.:

Experimental

5 Example 1: Synthesis of CRF Peptides

CRF peptides can be synthesized using well known Merrifield solid phase peptide synthesis technology. Specifically, the first carboxy-terminus amino acid of the CRF peptide sequence is attached to an insoluble polystyrene (benzhydrylamine derivative) resin by an amine bond and the remaining
10 amino acids of the peptide are subsequently linked in sequence. The amino acid residues are chemically protected to protect against undesirable coupling reactions between reactive side chains of the amino acid subunits. Once synthesis of the peptide is complete, liquid hydrogen fluoride is used to cleave the peptide from the resin and to deprotect the previously
15 protected reactive sites on the amino acid subunits.

Once completely synthesized, the crude CRF peptide is then purified by reverse-phase chromatography using a C-18 resin and an acetic acid/acetonitrile buffer system. Purer fractions are pooled for further purification using C-18 semi-preparative chromatography. Elution from this
20 column uses an acetic acid/acetonitrile and/or an ammonium bicarbonate/acetonitrile buffer system. The purest fractions are pooled and lyophilized. The purified peptide is then stored in glass containers at -25°C to -15°C until ready for the preparation of a liquid formulation thereof.

25 Example 2: Preparation of Liquid Formulations of CRF from Synthesized Peptide

From the solid CRF peptide synthesized in Example 1 above, a pH 4.0 liquid formulation of 1 mg/ml CRF in 20 mM acetate buffer and 5% mannitol was prepared as follows. Solid CRF peptide is removed from freezer

storage and is allowed to equilibrate at room temperature for at least 30 minutes. While the CRF peptide is equilibrating, 1,100 ml of sterile water and 1.38 ml glacial acetic acid is added to a sterile 2-liter flask and the solution is mixed for a minimum of 5 minutes. The solution is then brought
5 up to 1,200 ml with sterile water and mixed for at least an additional 5 minutes. This forms the "acetic acid" solution.

While the acetic acid solution is mixing, 1.088 grams of sodium acetate trihydrate is dissolved in 300 ml of sterile water, the solution is brought up to 400 ml with sterile water and is mixed for at least 5 minutes. This forms the
10 "sodium acetate" solution. Then, in a sterile 2-liter flask, 1,100 ml of the acetic acid solution and 240 ml of the sodium acetate solution are combined, mixed for a minimum of 5 minutes and the pH is then adjusted to 4.0 using the remaining acetic acid solution to lower the pH or the remaining sodium acetate solution to raise the pH. This solution is then sparged with
15 filtered nitrogen for at least 30 minutes. This forms the "buffer" solution.

Once sparged with nitrogen for at least 30 minutes, 800 ml of the buffer solution is transferred to a 2-liter graduated cylinder and 55 grams of mannitol is added. This solution is then mixed for at least 10 minutes until the mannitol is completely dissolved and while continuing to sparge the
20 solution with nitrogen. The pH of this solution is then adjusted to 4.0 by adding either the acetic acid solution to lower the pH or the sodium acetate solution to raise the pH. This forms the "buffer/mannitol" solution.

Once the buffer/mannitol solution is adjusted to pH 4.0, 1.1 grams (net peptide weight) of solid CRF is added and the solution is mixed until the
25 CRF is completely dissolved. A nitrogen flush is maintained over the solution during this time. Once the added CRF is completely dissolved, the volume of the solution is brought up to 1,100 ml with buffer/mannitol solution and mixed while maintaining a nitrogen flush over the solution. This forms the "CRF/buffer/mannitol" solution.

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The CRF/buffer/mannitol solution is then filtered through a sterile 0.22 micron pore size Durapore membrane filter while a nitrogen flush is maintained over the filtered solution. Sterile storage vials are then flushed with filtered nitrogen and 0.5 ml to 2.0 ml of the CRF/buffer/mannitol solution is aliquotted into vials which are then stoppered, sealed and stored at 2°-8°C. The resulting liquid formulation is 1.0 mg/ml CRF in 20 mM acetate buffer, pH 4.0 and 5% mannitol.

Example 3: Solubility of CRF in Various Buffer Solutions

In an effort to develop liquid formulations of CRF useful for commercial pharmaceutical purposes, experiments designed to measure the solubility of CRF in a variety of different buffer solutions were carried out. Specifically, CRF was added in increasing amounts to a variety of different buffer solutions at various different concentrations and pH's, either in the presence or absence of other excipient additives and the solubility of the CRF product was determined. Table 1 summarizes the results of these experiments measuring the solubility of CRF in various phosphate, citrate and acetate buffers, either in the presence or absence of various excipients such as NaCl and mannitol. CRF solubility is expressed in gross weight of CRF in mg/ml of solution.

The CRF/buffer/mannitol solution is then filtered through a sterile 0.22 micron pore size Durapore membrane filter while a nitrogen flush is maintained over the filtered solution. Sterile storage vials are then flushed with filtered nitrogen and 0.5 ml to 2.0 ml of the CRF/buffer/mannitol solution is aliquotted into vials which are then stoppered, sealed and stored at 2°-8°C. The resulting liquid formulation is 1.0 mg/ml CRF in 20 mM acetate buffer, pH 4.0 and 5% mannitol.

Example 3: Solubility of CRF in Various Buffer Solutions

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Table 1: Solubility of CRF in Various Buffer Solutions

	<u>pH</u> <u>(mg/ml)</u>	<u>BUFFER</u>	<u>SALT</u>	<u>ALCOHOL</u>	<u>SOLUBILITY</u>
5	2.0	10mM PO4	138mM NaCl		50
	3.6	10mM PO4	138mM NaCl		2.64
	4.2	10mM PO4	138mM NaCl		0.29
	4.6	10mM PO4	138mM NaCl		0.62
	5.6	10mM PO4	138mM NaCl		0.31
10	6.7	10mM PO4	138mM NaCl		0.62
	7.1	10mM PO4	138mM NaCl		0.48
	7.1	10mM PO4	138mM NaCl		0.84
15	6.02	5mM PO4			0.08
	6.86	5mM PO4			0.47
	7.7	5mM PO4			0.48
	7.32	20mM PO4			1.48
20	4.0	25mM Citrate	150mM NaCl		0.02
	4.0	25mM Citrate			0.04
	5.1	25mM Citrate	150mM NaCl		0.01
	5.1	25mM Citrate			0.02
	6.1	25mM Citrate	150mM NaCl		0.07
	6.1	25mM Citrate			0.1
25					
	4.08	5mM Citrate	150mM NaCl		0.16
	4.08	5mM Citrate			0.2
	4.92	5mM Citrate	150mM NaCl		0.01
	4.92	5mM Citrate			0.03
30	5.84	5mM Citrate	150mM NaCl		0.02
	5.84	5mM Citrate			0.04
35	4.2	25mM HOAc			29
	4.49	25mM HOAc	150mM NaCl		0.28
	4.49	25mM HOAc			5.34
	4.9	25mM HOAc	150mM NaCl		0.14
	4.9	25mM HOAc			0.37
	6.24	25mM HOAc	150mM NaCl		0.06
	6.24	25mM HOAc			0.09
40					
	4.2	5mM HOAc	150mM NaCl		1.17
	4.4	5mM HOAc			33
	4.8	5mM HOAc			2.2
45	4.7	5mM HOAc		5% mannitol	1.98
	4.72	5mM HOAc		1% mannitol	1.97
	4.81	5mM HOAc		1% Tween	1.13
	5.0	5mM HOAc			0.13
	6.0	5mM HOAc			0.03
50	4.28	20mM HOAc		5% mannitol	14.5
	4.34	20mM HOAc		5% mannitol	11.2
	4.45	20mM HOAc		5% mannitol	13.8
	4.52	20mM HOAc		5% mannitol	12.5
	4.73	20mM HOAc		5% mannitol	1.53
55	4.94	20mM HOAc		5% mannitol	0.07

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5.23 20mM HOAc 5% mannitol 0.04
The results presented in Table 1 demonstrate that CRF is very soluble in phosphate buffer between pH 2 and 3.6 and becomes less soluble between pH 5 and 6. The solubility of CRF then appears to increase when the pH is raised to greater than or equal to 7.0.

The results presented in Table 1 further demonstrate that CRF is very insoluble (≤ 0.2 mg/ml) in citrate buffer between pH 4 and 6.7 ,

Finally, CRF is very soluble in acetate buffer between pH 4 and 5 without added NaCl whereas addition of 150 mM NaCl acts to significantly reduce CRF solubility in acetate buffer. The addition of 5% mannitol does not appear to detrimentally affect the solubility of CRF at any pH tested.

Next, because it is known that certain buffers may be volatilized when lyophilized, further experiments were conducted as described above to examine the solubility of CRF in various other non-volatile buffer solutions. The results of these experiments are presented in Table 2.

Table 2: Solubility of CRF In Various Non-Volatile Buffer Solutions		
	<u>Buffer Solution</u>	<u>CRF solubility (net mg/ml)</u>
	25 mM Ascorbic, pH 4.0	4.5
	25 mM Lactic, pH 4.0	1.9
20	25 mM Succinic, pH 4.0	4.2
	25 mM Tartaric, pH 4.0	0.2
	10 mM Phosphate, pH 7.3	3.0
	10 mM Phosphate, pH 7.3 + 0.25% arginine	2.0
	10 mM Phosphate, pH 7.3 + 25 mM ascorbate	1.2
25	10 mM Phosphate, pH 7.3 + 0.25% glycine	2.2

The results presented in Table 2 demonstrate that CRF is extremely soluble in the ascorbic, lactic, succinic and phosphate buffers tested. Solubility was significantly reduced in the tartaric buffer tested.

Example 4: CRF Stability Studies In Ascorbic-, Succinic- and Phosphate-Based Buffers

Based upon the results presented in Table 2 demonstrating that CRF is very soluble in ascorbic-, succinic- and phosphate-based buffers, formulations having different concentrations of CRF were prepared in each of the three buffers and the rate of degradation of the CRF peptide in those formulations was determined after storage for 5 days at either 4°C or 30°C. In each formulation, buffer salts were set at 20 mM and mannitol was present at 5%. The rate of degradation was determined by measuring the concentration of CRF in each formulation prior to storage and again after 5 days in storage at each temperature examined. The results of these experiments are presented in Table 3.

Table 3: Stability of CRF in Ascorbic-, Succinic- and Phosphate-Based Buffers

Formulation	<u>Measured CRF Concentration (mg/ml)</u>		
	<u>time = 0</u>	<u>4°C, 5 days</u>	<u>30°C, 5 days</u>
Ascorbic, pH 4.0 (1 mg/ml CRF)	0.94	0.90	0.61
Ascorbic, pH 4.0 (0.5 mg/ml CRF)	0.56	0.52	0.35
Ascorbic, pH 4.0 (0.2 mg/ml CRF)	0.27	0.15	0.06
Succinic, pH 4.0 (1 mg/ml CRF)	0.98	1.02	0.99
Succinic, pH 4.0 (0.5 mg/ml CRF)	0.49	0.49	0.51
Succinic, pH 4.0 (0.2 mg/ml CRF)	0.22	0.21	0.21
Phosphate, pH 7.4 (1 mg/ml CRF)	1.00	1.01	0.97
Phosphate, pH 7.4 (0.5 mg/ml CRF)	0.42	0.41	0.41
Phosphate, pH 7.4 (0.2 mg/ml CRF)	0.18	0.15	0.15

The results presented in Table 3 demonstrate that while the ascorbic-based formulation is detrimental to the stability of CRF at both 4°C and 30°C, the succinic-based and phosphate-based formulations did not result in any significant degradation of the solublized CRF peptide.

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Example 6: Studies Analyzing the Effect of Mannitol and Sorbitol on CRF Stability in Liquid Formulations

Experiments were carried out to determine the effect of 5% mannitol or 5% sorbitol on the stability of liquid formulations of CRF during long-term storage at 4°C or 30°C. Specifically, the following liquid formulations were prepared and analyzed for stability during storage at either 4°C or 30°C:

- 1 mg/ml CRF, pH 4.0 acetate (20 mM)
- 1 mg/ml CRF, pH 4.0 acetate (20 mM), 5% mannitol
- 1 mg/ml CRF, pH 4.0 acetate (20 mM), 5% sorbitol
- 10 1 mg/ml CRF, pH 4.5 acetate (20 mM)
- 1 mg/ml CRF, pH 4.5 acetate (20 mM), 5% mannitol
- 1 mg/ml CRF, pH 4.5 acetate (20 mM), 5% sorbitol

Stability of the peptide in each formulation tested was determined by analyzing each of the samples by HPLC at various timepoints and measuring the amount of peptide degradation that had occurred at each timepoint by calculating the area under the peak corresponding to CRF as compared to the total area under all peaks detected.

The results obtained from these experiments demonstrated that after 2-weeks in storage at either 4°C and 30°C, the pH 4.0 formulations were equally stable as the pH 4.5 formulations and, therefore, the pH 4.5 formulations were dropped from the study.

The results of the studies for the pH 4.0 formulations performed at 30°C are shown in Figure 2. Specifically, the data presented in Figure 2 indicate that after 2-weeks of storage at 30°C, the pH 4.0 mannitol-containing formulation showed a slight trend toward greater stability than the sorbitol-containing formulation. Both the mannitol-containing and sorbitol-containing formulations, however, were significantly more stable than the formulation lacking additive alcohol. This same pattern was consistently observed through 12 weeks of storage at 30°C. Thus, these data indicate that the

addition of an alcohol such as mannitol or sorbitol functions to provide protection against degradation of CRF when stored as a liquid at 30°C.

The results of the studies performed at 4°C are presented in Figure 3. Specifically, the data presented in Figure 3 indicate that both the mannitol-
5 containing and sorbitol-containing formulations were significantly more stable than the formulation lacking any additive alcohol at the 2-month, 3-month and 5-month timepoints. These results, therefore, demonstrate that the addition of an alcohol such as mannitol or sorbitol to liquid formulations of CRF functions to provide protection against degradation of CRF when stored
10 as a liquid at 4°C.

Example 6: Studies on the Stability of CRF During Storage

Independent batches of pH 4.0 liquid formulations containing 1 mg/ml CRF in 20 mM acetate buffer and 5% mannitol (batch numbers NT194004 and NT195004), of a pH 7.4 liquid formulation of 1 mg/ml CRF in 10 mM
15 phosphate buffer and 5% mannitol (batch number 17210394) and of a pH 7.4 liquid formulation of 0.5 mg/ml CRF in 10 mM phosphate buffer and 5% mannitol (batch number 29210894) were prepared and were examined for their stability after storage for various periods of time at 2°-8°C. Percent stability of the liquid formulations was measured by subjecting the samples
20 to HPLC analysis after storage for the desired period of time and determining the relative area under the peak corresponding to CRF as compared to the total area under all peaks detected. The results presented in Table 4 and Figures 4 and 5 indicate that liquid formulations of CRF in both acetate and phosphate buffers are relatively stable over extended
25 periods of time at 2°-8°C.

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Table 4: Stability of CRF in Acetate and Phosphate-Based Buffers

<u>Formulation/Batch Number</u>		<u>Last Timepoint</u>	<u>Percent Stability</u>
Acetate/NTI94004		12 months	95%
Acetate/NTI95004		12 months	98%
5	Phosphate/172I0394	12 months	85%
	Phosphate/292I0694	15 months	≥95%

Two independent batches of CRF-containing formulations (batches NTI-02-03191 and NTI-07-05122) were prepared, lyophilized, stored for varying periods of time at -20°C to -15°C, reconstituted in saline and tested for their percent stability after storage. Prior to lyophilization, the liquid formulations constituted 0.2 mg/ml CRF, 0.1% HCl and 10 mg/ml mannitol. After 3 years of storage in a lyophilized form at -20°C to -15°C, batch NTI-02-03191 was shown to be greater than 97% stable by HPLC analysis as described above. After 3-years of storage at -20°C to -15°C, batch NTI-07-05122 was shown to be greater than 95% stable by HPLC analysis as described above.

Example 7: Studies on Pharmaceutical Formulations

A series of studies were carried out to evaluate the effects of different materials on the stability over time of the CRF peptide in various different liquid formulations. Multiple independent sets of CRF liquid formulations were analyzed separately. In each set of liquid formulations, an initial pH 4.0 liquid formulation consisting of 1 mg/ml CRF in 20 mM acetate buffer and 5% mannitol ("control" formulation) was employed and various components were either added thereto ("added" in Table 5), or in some cases, were substituted for a certain component or components in the initial formulation ("sub for..." in Table 5). Each formulation in each set of formulations was then stored at 40°C and the degree of degradation of the CRF peptide was determined at various timepoints by HPLC analysis. Each separate set of formulations was run with a concurrent control liquid formulation.

The results of these experiments are presented in Table 5. The quantitative results shown in Table 5 are the results obtained from the most recent timepoint of each experiment wherein the final timepoints for each of Sets A-H varied from days to more than eight weeks. Both the assay and purity results are presented as percent relative to the concurrent control; assay is measured as CRF concentration against a reference standard and purity is measured as the purity of the CRF HPLC peak relative to that of the other detectable CRF-related peaks in the sample.

TABLE 5: Stability of CRF in Various Liquid Formulations at 40°C

10	<u>Modification</u>	<u>Concentration</u>	<u>Assay - end of study</u> <u>% of control</u>	<u>Purity - end of study</u> <u>% of control</u>
	<u>Set A</u>			
	METHANOL (sub for mannitol)	1M	99%	100%
15	EDTA (added)	0.05%	84%	87%
	SUCROSE (sub for mannitol)	5%	27%	70%
	GLYCEROL (sub for mannitol)	5%	87%	88%
	HISTIDINE (added)	16mM	64%	74%
	GLUTAMIC ACID (added)	12mM	95%	87%
20	<u>Set B</u>			
	MANNITOL (sub for mannitol)	1%	108%	88%
	MANNITOL (sub for mannitol)	10%	105%	100%
	SODIUM CHLORIDE (added)	1%	na	na
	ACETATE (sub for acetate)	5 mM	108%	100%
25	ACETATE (sub for acetate)	50 mM	102%	88%
	SUCCINATE (sub for acetate)	20 mM	108%	88%
	CYSTEINE (added)	8 mM	74%	76%
	METHIONINE (added)	11 mM	107%	100%
	ARGININE (added)	10 mM	112%	100%
30	LYSINE (added)	10 mM	102%	88%
	ZINC ACETATE (added)	1 mM	94%	97%
	CUPROUS CHLORIDE (added)	0.8 mM	8%	36%
	FERROUS CHLORIDE (added)	0.8 mM	87%	100%

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TABLE 5, continued

		<u>Concentration</u>	<u>Assay - End of Study</u> <u>% of control</u>	<u>Purity - End of study</u> <u>% of control</u>
	<u>Set C</u>			
5	EDTA (added)	1.2 mM	70%	79%
	METHIONINE (added)	8.8 mM	115%	104%
	ARGININE (added)	8.6 mM	109%	102%
	LYSINE (added)	8.8 mM	108%	101%
	GLUTAMIC ACID (added)	11 mM	111%	101%
10	<u>Set D</u>			
	SODIUM BISULFITE (added)	4.5 mM	na	na
	MAGNESIUM CHLORIDE (added)	1.2 mM	100%	99%
	BHT (added)	0.1 mM	93%	99%
	PEG (added)	6.80%	102%	95%
15	<u>Set E</u>			
	GLUTAMIC ACID (added)	50 mM	88%	97%
	GLUTAMIC ACID (added)	10 mM	88%	102%
	GLUTAMIC ACID (added)	2 mM	90%	103%
	METHIONINE (added)	50 mM	99%	105%
20	METHIONINE (added)	10 mM	99%	103%
	METHIONINE (added)	2 mM	99%	102%
	GLU + MET (added)	10 + 10mM	93%	102%
	<u>Set F</u>			
	FERRIC CHLORIDE (added)	1 mM	58%	103%
25	FERRIC CHLORIDE (added)	0.2 mM	88%	102%
	FERRIC CHLORIDE (added)	0.05 mM	92%	101%
	CUPRIC CHLORIDE (added)	1 mM	91%	100%
	CUPRIC CHLORIDE (added)	0.2 mM	94%	101%
	CUPRIC CHLORIDE (added)	0.05 mM	94%	99%
30	<u>Set G</u>			
	CUPROUS CHLORIDE (added)	1 mM	35%	78%
	CUPROUS CHLORIDE (added)	0.2 mM	71%	93%
	CUPROUS CHLORIDE (added)	0.05 mM	91%	98%
35	<u>Set H</u>			
	METHIONINE (added)	10 mM	108%	101%
	METHIONINE (added)	50 mM	110%	102%
	ACETATE (sub for acetate)	10 mM	91%	88%

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ACETATE (sub for acetate)	5 mM	84%	91%
METHIONINE ONLY	20 mM	128%	105%

TABLE 5, continued

5	<u>Concentration</u>		<u>Assay - End of Study</u>	<u>Purity - End of study</u>
			<u>% of control</u>	<u>% of control</u>
<u>Set I</u>				
pH 7.4 10mM PO ₄ /5% mannitol			na	95%
pH 7.4 10mM PO ₄ /5% mannitol 10mM Glu			na	88%
pH 7.4 10mM PO ₄ /5% mannitol 10mM Met			na	98%

10

na = not available

The results presented in Table 5 demonstrate that the addition of amino acids such as methionine, arginine, lysine and glutamic acid provides protection against degradation of the CRF peptide in a liquid formulation. It is believed that the free amino acids alter the mechanism and rate of degradation of the peptide. In the case of additive methionine, the amino acid acts as a substitute site for oxidation, thereby reducing the oxidation of CRF itself. Thus, additive methionine exhibits a protective effect on the CRF peptide.

Furthermore, addition of the potential stabilizers magnesium, cupric or zinc salts also may provide a stabilizing effect against degradation of CRF in a liquid formulation, where as the addition of cuprous chloride appears to have a detrimental effect on CRF stability.

The results presented in Table 5 also indicate that various alcohols such as methanol or glycerol can effectively substitute for mannitol in the liquid formulation without having any significant detrimental effect on stability of CRF and, furthermore, mannitol concentrations in the liquid formulation can be varied between 1% and 10% without having any significant detrimental effect. Moreover, the results presented in Table 5 demonstrate that use of methionine or a succinate-based buffer in place of the control acetate-based buffer has no significant detrimental effect on CRF stability in a liquid formulation.

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Finally, the results presented in Table 5 demonstrate that addition of an antioxidant compound such as butylated hydroxytoluene (BHT) has no significant detrimental effects on the stability of CRF in a liquid formulation.

Example 8: Studies to Determine the Effects of Additive Methionine on CRF Stability

5 Follow-up experiments were performed to confirm the protective effect provided by the addition of methionine to the CRF formulations of the present invention. Figure 6 shows the relative rate of degradation of the CRF peptide during storage at 40°C in a control liquid formulation (20 mM acetate and 5% mannitol) versus that wherein either 10 mM or 50 mM
10 methionine is added to the control formulation. The "area %" of the liquid formulations was measured by subjecting the samples to HPLC analysis after storage for the desired period of time and determining the relative area under the peak corresponding to CRF as compared to the total area under
15 all peaks detected.

The results presented in Figure 6 demonstrate that while the addition of 10 mM methionine to the CRF formulations provides for a small protective effect against degradation of the peptide at 40°C, addition of 50 mM methionine to the control formulation provides a significantly increased
20 protective effect.

Figure 7 shows the stability of CRF at 40°C in the presence of various acetate and/or methionine concentrations. The results presented in Figure 7 demonstrate that the substitution of 20 mM methionine in place of acetate, provides a significant protective effect as compared to that control
25 formulation.

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Finally, Figure 8 also demonstrates that the substitution of either 20 mM or 50 mM methionine in place of acetate in formulations of the CRF peptide at 40°C acts to significantly decrease the rate at which the CRF peptide is degraded.

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Example 9: Studies to Determine the Stability of CRF as a Lyophilized Powder Versus as a Reconstituted Liquid

Experiments were performed to determine the difference in CRF stability when stored as a lyophilized power ("powder" form) versus when stored in a liquid formulation that has been reconstituted from that lyophilized powder } *urad* ~~urad~~ ("liquid" form). For these experiments, two different CRF formulations were prepared as lyophilized powders and a portion of each was reconstituted as a liquid. Both the powder and liquid forms of the peptide were then stored over a period of 6 weeks at either 30°C or 40°C to determine the amount of CRF degradation that occurred during the storage period. The formulations tested were (1) 0.2 mg/ml CRF in 10 mM succinate, pH 4.0 and 5% mannitol ("succinate" formulation) and (2) 0.2 mg/ml CRF in 10 mM phosphate buffer, pH 7.4 and 5% mannitol ("phosphate" formulation). The results of these experiments are presented in Table 6. The numbers presented in Table 6 represent the % of the initial CRF concentration at each timepoint analyzed. "ND" means that concentration measurements were not performed at those timepoints.

Table 6: Stability of Various Liquid and Powder Formulations of CRF

<u>Formulation/Form</u> <u>wks</u>	<u>Temp</u>	<u>CRF concentration (% of initial)</u>					
		<u>0 days</u>	<u>4 days</u>	<u>7 days</u>	<u>14 days</u>	<u>4 wks</u>	<u>6</u>
Succinate/Powder	30°C	100	ND	ND	83		101
75							
Succinate/Powder	40°C	100	ND	ND	63		59
32							
Phosphate/Powder	30°C	100	ND	ND	90		103
76							
Phosphate/Powder	40°C	100	ND	ND	58		32
66							
Succinate/Liquid	30°C	100	92	93	100	121	ND

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Phosphate/Liquid 30°C 100 91 98 92 107 ND

The data presented in Table 6 indicate that liquid solutions of CRF seem to be as stable over time as the lyophilized powders of CRF. These data are surprising in light of the general acceptance in the art that lyophilized
5 formulations are more stable over time than are their liquid counterparts.

Example 10: Subcutaneous and Intracutaneous Irritation
Screening

Experiments were performed to determine the irritation potential of various formulations which either possess or lack the CRF peptide. First, two CRF-
10 containing formulations were prepared and administered subcutaneously as a single dose to rats and the degree of irritation at the administration site was observed at various timepoints following administration. The irritation potential of the following formulations was analyzed:

- 1 mg/ml CRF, pH 4.0, 20 mM acetate buffer, 5% mannitol
- 15 1 mg/ml CRF, pH 4.0, 20 mM acetate buffer, 5% sorbitol

The results of these experiments demonstrated that both of the formulations analyzed were non-irritating to the test animals when administered as a single dose subcutaneously. When administered subcutaneously daily for seven days to rats, no irritation was observed visually but the formulations
20 demonstrated irritation potential upon microscopic examination of the injection site.

Next, the irritation potential of various liquid vehicles lacking additive CRF was examined in a rabbit intracutaneous irritation model. In this model, the backs of test rabbits are shaved, 0.2 ml of each test formulation vehicle is
25 injected intracutaneously at five different sites on the back of the rabbit and the injection sites are observed for the presence of erythema/eschar formation and edema at 24, 48 and 72 hours after injection. At each

observational time point, erythema/eschar formation is scored on a scale of 0 to 4 as shown in Table 7.

Table 7: Scoring of Erythema and Eschar Formation

	<u>Score</u>
5 No erythema	
0	
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
10 Severe erythema (beet-redness) to slight eschar formation	4

Furthermore, at each observational time point, edema formation, excluding non-inflammatory (mechanical) edema from the blank or extraction fluid, is scored on a scale of 0 to 4 as shown in Table 8.

Table 8: Scoring of Edema Formation

	<u>Score</u>
15 No edema	0
Very slight edema (barely perceptible)	1
Light edema (edges of area well defined by definite raising)	
2	
20 Moderate edema (raised about 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4

Upon completion of the studies, a "mean irritation score" is determined for each formulation tested by adding the erythema/eschar formation score and
25 the edema score at each of the five injection sites and at each of the three different observational time points and dividing that total score by the number of scores taken at each site and at each observational time point (2)

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multiplied by the number of injection sites observed at each time point (5) multiplied by the number of observational time points (3). The mean irritation scores of various different CRF formulation vehicles are shown in Table 9.

5 **Table 9: Mean Irritation Scores for Various CRF Vehicle Solutions**

<u>Test Vehicle</u>	<u>Mean irritation score</u>
Saline control	0
pH 4.0, 20 mM NaOAc buffer	2.9
pH 4.0, 20 mM NaOAc buffer, 5% mannitol	0.8
10 pH 4.0, 5 mM NaOAc buffer, 5% mannitol	0
pH 7.4, 10 mM PO ₄ buffer	0.8
pH 7.4, 10 mM PO ₄ buffer, 5% mannitol	0

These results demonstrate that while the pH 4.0, 20 mM acetate buffer vehicle exhibits a relatively high mean irritation score, addition of an alcohol
 15 such as mannitol to that vehicle has the effect of significantly reducing the irritative effect of the liquid formulation. The same protective effect is observed when mannitol is employed in association with a phosphate buffer at pH 7.4.

Similar experiments were performed to determine the effects of additive
 20 methionine on the irritation potential of a CRF formulation. The results of the experiments testing various different formulation vehicles are shown in Table 10.

Table 10: Mean Irritation Scores for Various CRF Vehicle Solutions

<u>Test Vehicle</u>	<u>Mean irritation score</u>
25 Saline control	0
pH 4.0, 20 mM NaOAc buffer, 5% mannitol	2.3

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	pH 4.0, 10 mM NaOAc buffer, 5% mannitol	0.8
	pH 4.0, 5 mM NaOAc buffer, 5% mannitol	0.3
	pH 4.0, 5 mM NaOAc buffer, 5% mannitol, 20 mM methionine	0.6
	pH 4.0, 5 mM NaOAc buffer, 5% mannitol, 50 mM methionine	0
5	pH 4.0, 20 mM methionine, 5% mannitol	0
	pH 4.0, 50 mM methionine, 5% mannitol	0

The results presented in Table 10 demonstrate that methionine-containing formulations are generally less irritating than similar formulations lacking methionine. Also, substitution of methionine for acetate results in a
10 formulation which is less irritating.

The foregoing description of the preferred embodiments of the present invention has been presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise form disclosed, and many modifications and variations are possible
15 in light of the above teaching. Such modifications and variations which may be apparent to a person skilled in the art are intended to be within the scope of the invention.

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WHAT IS CLAIMED IS:

1. A pharmaceutically acceptable formulation of corticotrophin releasing factor (CRF) comprising (a) a pharmaceutically effective amount of CRF, (b) a buffer at a concentration of from about 1 mM to about 50 mM
5 which functions to maintain the pH of said formulation between about 2.0 to about 5.0 or between about 6.0 to about 9.0 when said formulation is in liquid form, wherein the anion of said buffer is selected from the group consisting of acetate, phosphate, carbonate, succinate, citrate, borate, tartrate, fumarate and lactate and (c) an alcohol, in a concentration of from
10 about 1% to about 10%, wherein said alcohol is selected from the group consisting of mannitol, sorbitol, ribitol, arabitol, xylitol, inositol, galactitol, methanol, ethanol and glycerol.
2. The formulation according to Claim 1 further comprising an amino acid selected from the group consisting of methionine, arginine,
15 lysine, cysteine, glutathione and glutamic acid in a concentration from about 1 mM to about 100 mM.
3. The formulation according to Claim 2 wherein the amino acid is methionine.
4. The formulation according to Claim 3 wherein said methionine
20 is at a concentration from about 10 mM to about 50 mM:
5. The formulation according to Claim 1 further comprising a sugar in a concentration from about 1% to about 10%.
6. The formulation according to Claim 5 wherein the sugar is selected from the group consisting of glucose, sucrose, lactose, fructose,
25 trehalose and mannose.
7. The formulation according to Claim 1 further comprising a ferric or cupric salt in a concentration from about 0.05 mM to about 1 mM.

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8. The formulation according to Claim 7 wherein said ferric or cupric salt is ferric chloride or cupric chloride.
9. The formulation according to Claim 1 further comprising butylated hydroxytoluene in a concentration from about 0.05 mM to about
5 0.5 mM.
10. The formulation according to Claim 1 further comprising magnesium chloride or zinc chloride in a concentration from about 0.5 mM to 2.0 mM.
11. The formulation according to Claim 1 further comprising any
10 chemically compatible preservative.
12. The formulation according to Claim 11 wherein said chemically compatible preservative is selected from the group consisting of benzyl alcohol, chlorobutanol, phenol, cresol, methyl paraben and propyl paraben.
13. The formulation according to Claim 1 wherein said formulation
15 is 0.5-3 mg/ml CRF in 20 mM acetate buffer and 5% mannitol at pH 4.0.
14. The formulation according to Claim 1 wherein said formulation is 0.5-3 mg/ml CRF in 20 mM acetate buffer, 5% mannitol and 50 mM methionine at pH 4.0.
15. A pharmaceutically acceptable formulation of corticotrophin
20 releasing factor (CRF) comprising (a) a pharmaceutically effective amount of CRF, (b) an amino acid at a concentration of from about 1 mM to about 100 mM, wherein said amino acid is selected from the group consisting of methionine, arginine, lysine, cysteine, glutathione and glutamic acid and (c)
25 an alcohol, in a concentration of from about 1% to about 10%, wherein said alcohol is selected from the group consisting of mannitol, sorbitol, ribitol, arabitol, xylitol, inositol, galactitol, methanol, ethanol and glycerol.

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16. The formulation according to Claim 15 wherein said amino acid is methionine.
17. The formulation according to Claim 16 wherein said methionine is present at a concentration of from about 10 mM to about 50 mM.
- 5 18. The formulation according to Claim 17 wherein said methionine is present at a concentration of about 20 mM.
19. The formulation according to Claim 15 wherein said formulation is 0.5-3 mg/ml CRF in 20 mM methionine and 5% mannitol at pH 4.0.
20. The formulation according to Claim 15 further comprising a
10 chemically compatible preservative.
21. The formulation according to Claim 20 wherein said chemically compatible preservative is selected from the group consisting of benzyl alcohol, chlorobutanol, phenol, cresol, methyl paraben and propyl paraben.
22. The formulation according to Claims 1 or 15 in liquid form.
- 15 23. The formulation according to Claims 1 or 15 in lyophilized form.
24. A method of preparing a pharmaceutically acceptable formulation of corticotropin releasing factor peptide comprising dissolving said corticotropin releasing factor peptide in a solution comprising (a) either
20 (i) a buffer at a concentration of from about 1 mM to about 50 mM which functions to maintain the pH of said formulation between about 2.0 to about 5.0 or between about 6.0 to about 9.0 when said formulation is in liquid form, wherein the anion of said buffer is selected from the group consisting of acetate, phosphate, carbonate, succinate, citrate, borate, tartrate, fumarate and lactate or (ii) an amino acid at a concentration of from about 1
25 mM to about 100 mM, wherein said amino acid is selected from the group

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consisting of methionine², arginine, lysine, cysteine, glutathione and glutamic acid and (b) an alcohol in a concentration of from about 1% to about 10%, wherein said alcohol is selected from the group consisting of mannitol, sorbitol, ribitol, arabitol, xylitol, inositol, galactitol, methanol, ethanol and glycerol.

25. The method according to Claim 24 wherein when said solution comprises said buffer, the further step of adding to said formulation an amino acid selected from the group consisting of methionine, arginine, lysine, cysteine, glutathione and glutamic acid in a concentration from about 1 mM to about 100 mM.

26. The method according to Claim 24 further comprising the step of adding to said formulation a sugar in a concentration from about 1% to about 10%.

27. The method according to Claim 26 wherein the sugar is selected from the group consisting of glucose, sucrose, lactose, fructose, trehalose and mannose.

28. The method according to Claim 24 further comprising the step of adding to said formulation a ferric or cupric salt in a concentration from about 0.05 mM to about 1 mM.

29. The method according to Claim 28 wherein said ferric or cupric salt is ferric chloride or cupric chloride.

30. The method according to Claim 24 further comprising the step of adding to said formulation butylated hydroxytoluene in a concentration from about 0.05 mM to about 0.5 mM.

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31. The method according to Claim 24 further comprising the step of adding to said formulation magnesium chloride or zinc chloride in a concentration from about 0.5 mM to 2.0 mM.
32. The method according to Claim 24 further comprising adding a
5 chemically compatible preservative.
33. The method according to Claim 32 wherein said chemically compatible preservative is selected from the group consisting of benzyl alcohol, chlorobutanol, phenol, cresol, methyl paraben and propyl paraben.
34. The method according to Claim 24 further comprising the step
10 of introducing said formulation into its storage container under inert gas.
35. The method according to Claim 24 further comprising the step of lyophilizing said formulation.
36. A method for enhancing the stability of corticotropin releasing factor in a formulation thereof comprising adding to said formulation an
15 amino acid selected from the group consisting of methionine, arginine, lysine, cysteine, glutathione and glutamic acid in a concentration from about 1 mM to about 100 mM.
37. The method according to Claim 36 wherein said amino acid is methionine.
- 20 38. A method for reducing inflammation or edema in a mammal, said method comprising administering to said mammal a therapeutically effective amount of the formulation according to Claims 1 or 15.
39. The method according to Claim 38 wherein said formulation is administered by the intravenous, subcutaneous, intramuscular, nasal or
25 pulmonary routes of administration.

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Internationales Büro
**INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE
INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)**

(51) Internationale Patentklassifikation ⁶: A61K 38/21, 9/08	A1	(11) Internationale Veröffentlichungsnummer: WO 99/15193 (43) Internationales Veröffentlichungsdatum: 1. April 1999 (01.04.99)
(21) Internationales Aktenzeichen: PCT/EP98/06065 (22) Internationales Anmeldedatum: 23. September 1998 (23.09.98) (30) Prioritätsdaten: 97116562.6 23. September 1997 (23.09.97) EP (71) Anmelder (für alle Bestimmungsstaaten außer US): DR. RENTSCHLER BIOTECHNOLOGIE GMBH [DE/DE]; Erwin-Rentschler-Strasse 21, D-88471 Laupheim (DE). (72) Erfinder; und (73) Erfinder/Anmelder (nur für US): TSCHÖPE, Michael [DE/DE]; Kastanienweg 72, D-88400 Biberach (DE). SIKLOSI, Thomas [DE/DE]; Alpenweg 15, D-88487 Walpertshofen (DE). SCHROEDER, Peter [DE/DE]; Hopfenweg 17, D-88471 Laupheim (DE). HOFER, Hans [DE/DE]; Beim Klippels 11, D-88487 Walpertshofen (DE). (74) Anwälte: WEICKMANN, H. usw.; Kopernikusstrasse 9, D-81679 München (DE).		(81) Bestimmungsstaaten: AU, CA, IL, JP, KR, US, europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Veröffentlicht <i>Mit internationalem Recherchenbericht. Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist; Veröffentlichung wird wiederholt falls Änderungen eintreffen.</i>
(54) Title: LIQUID INTERFERON- β FORMULATIONS (54) Bezeichnung: FLÜSSIGE INTERFERON- β -FORMULIERUNGEN (57) Abstract The invention relates to liquid formulations of human interferon-β. The inventive formulations are characterised in that they have a buffer with a pH value in the slightly acidic to neutral range between 5 and 8, preferably between 5.5 and 8, and in that the interferon-β is highly stable in solution, the molecular integrity being preserved. (57) Zusammenfassung Die vorliegende Erfindung betrifft flüssige Formulierungen von humanem Interferon-β. Die Formulierungen sind dadurch gekennzeichnet, daß sie einen Puffer mit einem pH-Wert im schwach sauren bis neutralen Bereich zwischen 5 und 8, bevorzugt zwischen größer 5,5 und 8 aufweisen sowie eine hohe Stabilität des Interferon-β in Lösung unter Beibehalt der molekularen Integrität gegeben ist.		

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165**Patentansprüche**

1. Flüssige Formulierung, die humanes Interferon- β als Wirkstoff in einer
Konzentration bis zu 25 MU/ml und einen Puffer zur Einstellung eines
pH-Werts von 5 bis 8 enthält, frei von Humanserumalbumin ist und
eine Langzeitstabilität der biologischen Aktivität (in vitro) von
mindestens 80% der Ausgangsaktivität nach Lagerung bei 25°C für
3 Monate aufweist.
2. Flüssige Formulierung, die humanes Interferon- β als Wirkstoff und
einen Puffer zur Einstellung eines pH-Werts von 6 bis 7,2 enthält, frei
von Humanserumalbumin ist und eine Langzeitstabilität der biologi-
schen Aktivität (in vitro) von mindestens 80% der Ausgangsaktivität
nach Lagerung bei 25°C für 3 Monate aufweist.
3. Flüssige Formulierung, die humanes Interferon- β als Wirkstoff, einen
Puffer zur Einstellung eines pH-Werts von 5 bis 8 und eine oder
mehrere Aminosäuren enthält und eine Langzeitstabilität der biologi-
schen Aktivität (in vitro) von mindestens 80% der Ausgangsaktivität
nach Lagerung bei 25°C für 3 Monate aufweist.
4. Formulierung nach Anspruch 1,
dadurch gekennzeichnet,
daß sie ein glykosiliertes Interferon- β enthält.
5. Formulierung nach Anspruch 2,
dadurch gekennzeichnet,
daß das Interferon- β aus CHO-Zellen stammt.

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6. Formulierung nach einem der Ansprüche 1 bis 5,
dadurch gekennzeichnet,
daß sie den Puffer in einer Konzentration von 10 mmol/l bis 1 mol/l
enthält.
- 5 7. Formulierung nach einem der Ansprüche 1 bis 6,
dadurch gekennzeichnet,
daß sie einen Puffer ausgewählt aus der Gruppe bestehend aus
Phosphat-, Citrat- und Acetatpuffern und Mischungen davon enthält.
- 10 8. Formulierung nach Anspruch 7,
dadurch gekennzeichnet,
daß sie einen Phosphat/Citratpuffer enthält.
- 15 9. Formulierung nach einem der Ansprüche 1 und 3 bis 8,
dadurch gekennzeichnet,
daß sie einen pH-Wert zwischen 6 und 7,2 aufweist.
- 20 10. Formulierung nach Anspruch 3,
dadurch gekennzeichnet,
daß sie frei von Humanserumalbumin ist.
- 25 11. Formulierung nach einem der Ansprüche 1 bis 10,
dadurch gekennzeichnet,
daß sie abgesehen vom Wirkstoff frei von humanen oder tierischen
Polypeptiden ist.
- 30 12. Formulierung nach einem der Ansprüche 1 bis 11,
dadurch gekennzeichnet,
daß sie frei von oberflächenaktiven Verbindungen ist.

13. Formulierung nach einem der Ansprüche 1 bis 12,
dadurch gekennzeichnet,
daß sie eine chemische Integrität nach Lagerung bei 25°C für 6
Monate aufweist.
14. Formulierung nach einem der Ansprüche 1 bis 13,
dadurch gekennzeichnet,
daß sie eine physikalische Integrität nach Lagerung bei 25°C für 6
Monate aufweist.
15. Formulierung nach einem der Ansprüche 1, 2 und 4 bis 14,
dadurch gekennzeichnet,
daß sie weiterhin eine oder mehrere Aminosäuren enthält.
16. Formulierung nach Anspruch 3 oder 15,
dadurch gekennzeichnet,
daß sie Methionin enthält.
17. Formulierung nach Anspruch 16,
dadurch gekennzeichnet,
daß das Methionin in einer Konzentration von 0,1 bis 4 mmol/l
vorliegt.
18. Formulierung nach einem der Ansprüche 1 bis 17,
dadurch gekennzeichnet,
daß sie weiterhin Hilfsstoffe zur Einstellung der Tonizität enthält.
19. Formulierung nach einem der Ansprüche 1 bis 18,
dadurch gekennzeichnet,
daß sie weiterhin Verdickungsmittel zur Viskositätserhöhung enthält.

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20. Formulierung nach einem der Ansprüche 1 bis 19,
dadurch gekennzeichnet,
daß sie weiterhin physiologisch verträgliche Konservierungsmittel
enthält.
- 5
21. Pharmazeutisches Präparat,
dadurch gekennzeichnet,
daß es eine flüssige Formulierung nach einem der Ansprüche 1 bis 20
enthält.
- 10
22. Pharmazeutisches Präparat nach Anspruch 21 zur oralen, parentera-
len oder ophthalmologischen Applikation.
23. Pharmazeutisches Präparat nach Anspruch 21 oder 22 mit Einzel-
15 dosen von 1 bis 25 MU.
24. Verfahren zur Herstellung eines pharmazeutischen Präparats nach
einem der Ansprüche 21 bis 23,
dadurch gekennzeichnet,
20 daß man eine Formulierung nach einem der Ansprüche 1 bis 20 und
gegebenenfalls weitere galenisch notwendige Hilfsstoffe zubereitet
und in eine geeignete Darreichungsform bringt.
- 25
25. Verfahren zur Verbesserung der Haltbarkeit einer flüssigen Formu-
26 lung, die humanes Interferon- β als Wirkstoff und einen Puffer zur
Einstellung eines pH-Werts von 5 bis 8 enthält,
dadurch gekennzeichnet,
daß man eine Formulierung ohne Humanserumalbumin oder/und mit
einer oder mehreren Aminosäuren verwendet.
- 30