SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 07-009315- -00002-0000

Fecha: 2008-01-29 13:41:46 Dep: 2020 NUECREACIONI
Tra: 11 PATENTE(YII Eve: 378 FASENACIONALI
Act: 400 OPOSIOBSERVTER Folios: 129

Industria y Comercio
SUPERINTENDENCIA

FORMULARIO ÚNICO DE OPOSICIÓN

① OPOSICIÓN A :

- [X] Patente de Invención.
- [ ] Patente de Modelo de Utilidad.
- [ ] Diseño Industrial.
- [ ] Esquema de Trazado para Circuitos Integrados.
- [ ] Marcas de Productos o Servicios.
- [ ] Marca Colectiva.
- [ ] Marca de Certificación.
- [ ] Lema Comercial.

② SOLICITUD PUBLICADA

Número del expediente: 07-009315
Solicitante: WYETH
Apoderado: ALICIA LLOREDA RICAURTE
Título de la nueva creación o denominación del signo: "FORMULACIONES PARA ALMACENAR PROTEÍNAS.".
Gaceta: 581

③ OPOSICIÓN PRESENTADA POR:

SOLICITANTE	Nombre: PROCAPS S.A Dirección: CALLE 80 No. 78B -201 Nacionalidad o Domicilio: COLOMBIA Lugar de Constitución: BARRANQUILLA Teléfono: (571)3719900 Fax: (571)3730818 E-mail:	**IDENTIFICACIÓN** C.C. [ ] NIT [X] C.E. [ ] Otro [ ] Cual ____ Número 890.106.527-5
REPRESENTANTE O APODERADO	Nombre: ELIANA ISAURA MORENO BOHORQUEZ Dirección: CARRERA 13 No. 119-95 OF 104 Teléfono: Fax: E-mail: (571)2131213 (571)6121979 negocios@optimum-co.com	**IDENTIFICACIÓN** C.C. [X] NIT [ ] C.E. [ ] Otro [ ] Cual ____ Número 51.975.664 TP 83823

④ FUNDAMENTOS DE LA OPOSICIÓN:

Art.14, 16, 18, 20 y 30 de la Decisión 486 de la CAN

Causal: No cumple con los requisitos de patentabilidad.

Signo opositor:

Justificación:

La presente solicitud no cumple con los requisitos de patentabilidad dado que lo revelado, ya se encontraba en el estado de la técnica o es posible derivarlo de la misma tal y como se demostrará en el capítulo "Fundamento de Hechos".

⑤ **Comprobante de pago No.** _____ **Fecha** _____

⑥ **ANEXOS**

- [x] Comprobante de pago de la tasa.
- [x] Poderes, si fuere el caso.
- [x] Pruebas de los fundamentos de la oposición.
- [ ] Documentos que acrediten el legítimo interés, si fuere el caso.
- [x] Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas.

⑦

NOMBRE: **ELIANA ISAURA MORENO BOHORQUEZ**

FIRMA:

C.C. 51.975.664 **TP.** 83823

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

Señora Jefe,

DIVISIÓN DE NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D.

Referencia	**: Expediente N° 07 009315**
Asunto	**: Oposición a la solicitud de patente de Invención titulada "FORMULACIONES PARA ALMACENAR PROTEÍNAS."**
Solicitante	**: WYETH**
Opositora	**:PROCAPS S.A.**

Publicada en la Gaceta de la Propiedad Industrial

N° 581 del 31 de octubre de 2007

Yo, ***ELIANA ISAURA MORENO BOHORQUEZ***, abogada en ejercicio, identificada como aparece al pie de su firma, con tarjeta profesional No. 83823 del Consejo Superior de la Judicatura, en mi condición de apoderada de la sociedad ***PROCAPS S.A.*** conformada bajo las leyes de la República de Colombia y domiciliada en la ciudad de Barranquilla, Colombia, atentamente manifiesto que estando dentro del término de ley y por orden expresa de mi representada, presento oposición a la concesión del registro de la Patente de Invención titulada **"FORMULACIONES PARA ALMACENAR PROTEÍNAS"**, cuyo titular es **WYETH,** y pido a Usted declarar fundada esta oposición y en consecuencia negar el registro de la patente solicitada.

1. INTERÉS PARA ACTUAR

Conforme a lo dispuesto en el artículo 42 de la Decisión 486 de 2000 de la CAN, estando dentro del término estipulado por la ley, manifiesto el legitimo interés de nuestra representada para oponerse al registro de la patente de la referencia, atendiendo a que la solicitud pretendida no cumple con los requisitos dispuestos en la ley como se analizará más adelante y de ser concedida afectaría directamente la comercialización de algunos productos de mi representada, que contienen, en particular formulaciones de proteínas. Por lo tanto, solicito respetuosamente a ese Despacho se sirva tener en cuenta la presente oposición dentro del trámite administrativo que se surte en esta Superintendencia.

2. FUNDAMENTOS DE HECHOS

2.1. La solicitud objeto de la presente fue presentada el 31 de enero de 2007, reivindicando prioridad bajo las solicitudes US20040601311P del 2004-08-13 y fue publicada en Colombia, en la Gaceta de la Propiedad Industrial N° 581 del 31 de octubre de 2007 página 467 y N° publicación 599, cuya publicación internacional corresponde a la WO2006020935 del 23/02/2006.

2.2. La solicitud colombiana **07-009315** objeto de la presente oposición, se refiere a una formulación, caracterizada porque comprende una proteína aislada; y una solución acuosa que tiene pH desde 4,0 hasta un pH de 8, en donde la formulación no contiene un crioprotector o surfactante, y la proteína es estable por al menos 3 semanas desde una temperatura de -80°C hasta 8°C; donde la proteína es un anticuerpo y la solución acuosa es agua estéril. De igual forma, la solicitud se refiere a un método para su almacenaje; un método para determinar la formulación favorable; y, un polipéptido producido y su uso en la manufactura de proteínas modificadas.

2.3.En primera instancia, se debe advertir que las reivindicaciones que se refieren al método de almacenaje no conforman unidad de invención pues nada tiene que ver una formulación farmacéutica con el método de almacenamiento. Son materias distintas y por lo tanto no conforman unidad de invención. Según la legislación, Decisión 486 de la Comunidad Andina de Naciones, Artículo 36.- "(...) *la oficina nacional competente podrá, en cualquier momento del trámite, requerir al solicitante que divida la solicitud si ella no cumpliera con el requisito de unidad de invención*".

2.4.Ahora bien, con base en el artículo 14 de la Decisión 486 de la CAN consagra lo siguiente: *"Los países miembros otorgarán patentes para las invenciones, sean de producto o procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial"*. Así las cosas, las invenciones objeto de patente deben ser nuevas y tener nivel inventivo. Bien, pues antes de la fecha de prioridad de esta solicitud se encuentran los siguientes documentos que afectan la patentabilidad de la presente solicitud:

- D1: WO9856418 publicada el 17 de diciembre de 1998.
- D2: US6267958 publicada el 31 de julio de 2001.

2.5.Ahora bien, el documento D1 particularmente en las reivindicaciones 1 a 22, páginas 4 a 10, enseña formulaciones acuosas de anticuerpos que comprende de 10 a 25 mM de acetato de sodio con un pH 6 en una concentración de 40 mg/ml. Dicho documento anterior D1 igualmente se refiere a anticuerpos monoclonales, humanizados, anticuerpos recombinantes, disponibles para propósitos terapéuticos, donde dicha formulación revelada es estable de 2 a 8 °C por al menos 2 años (página 6 de D1). De igual forma, esta anterioridad revela que estos anticuerpos son específicos como antígenos sanguíneos tal como Lewis Y o como receptores Erb tal como CD22. De igual forma, esta anterioridad revela que la

formulación de anticuerpos comprende NaCl y un método para estabilizar dicho anticuerpo en la página 24 a 46. Así, las cosas la presente solicitud no es novedosa pues lo revelado ya se encontraba en el estado de la técnica, particularmente en el documento D1.

2.6. El documento D2, según la descripción y particularmente la cláusulas 1 a 47 revela por su parte formulaciones acuosas de anticuerpos que comprenden de 10 a 25 mM de acetato de sodio con un pH 6 en una concentración de 30 mg/ml. Según dicho documento anterior D2 particularmente en la columna 6 a 13, también se refiere a anticuerpos monoclonales, humanizados, anticuerpos recombinantes, disponibles para propósitos terapéuticos, donde dicha formulación revelada es estable de 2 a 8 °C por al menos 2 años. De igual forma, esta anterioridad D2 anticipa que estos anticuerpos son específicos como antígenos sanguíneos (tal como Lewis Y) o como receptores Erb (tal como CD22) y agrega en la columna 14 a 24 que la formulación de anticuerpos comprende NaCl y un método para estabilizar dicho anticuerpo. Por lo tanto, este documento objeto de oposición a la luz del documento D2 carece evidentemente de NOVEDAD.

2.7. En consecuencia y de acuerdo a lo descrito anteriormente, se deriva que la solicitud colombiana **07 009315** no puede pretender un monopolio por patente toda vez que lo que pretende no cumple con uno de los tres requisitos necesarios para su protección.

2.8. Que por consiguiente, respecto a la solicitud pretendida se tiene que:

2.8.1. **CARECE DE NOVEDAD**: Según la decisión 486 de la Comunidad Andina de Naciones, Artículo 16.- *"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."*. A la fecha de prioridad, 13 de agosto de 2004 ya era

conocido, pues como se demostró, era parte del arte anterior con base en los documentos D1 y D2, una formulación que comprende una proteína aislada; y una solución acuosa que tiene pH desde 4,0 hasta un pH de 8, en donde la formulación no contiene un crioprotector o surfactante, y la proteína es estable por al menos 3 semanas desde una temperatura de -80°C hasta 8°C, sobre el cual busca protección en el capítulo reivindicatorio.

2.9. Dado lo anterior, es posible deducir que la solicitud y lo reivindicado no es objeto de patentabilidad pues es contrario a lo que dicta la Decisión 486 de la Comunidad Andina de Naciones, Artículo 14.- *"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."*. La solicitud pretendida no cumple con el requisito de NOVEDAD, uno de los tres requisitos necesarios para su patentabilidad.

2.10. Por todo lo anterior, atentamente solicito a usted que se sirva declarar fundada la presente oposición y negar el registro Patente de invención denominada **"FORMULACIONES PARA ALMACENAR PROTEÍNAS"**, cuyo titular es **WYETH,,** solicitud Colombiana que apareció publicada en la Gaceta de la Propiedad Industrial N° 581.

3. PRUEBAS

De conformidad con lo dispuesto por las normas vigentes, solicito a usted tener como prueba en este asunto los siguientes:

- D1: WO9856418 publicada el 17 de diciembre de 1998.

- D2: US6267958 publicada el 31 de julio de 2001.

4. FUNDAMENTO DE DERECHO

Fundamento esta observación en las normas legales siguientes:

4.1. Numeral 1 del artículo 586 del Código de Comercio.

4.2. Artículo 42 de la Decisión 486 de la Comisión del Acuerdo de Cartagena.

4.3. En las demás normas concordantes.

5. ANEXOS

Para los efectos correspondientes anexo al presente escrito los siguientes documentos:

1. Poder para actuar en el presente.
2. Documento que acredita al representante legal de PROCAPS S.A.
3. Recibo de caja del respectivo pago de tasa.
4. Copias de los documentos:

- D1: WO9856418 publicada el 17 de diciembre de 1998.
- D2: US6267958 publicada el 31 de julio de 2001.

6. NOTIFICACIONES

Para efectos de notificaciones que por disposición del artículo 50 del Decreto 01 de 1984 debe hacer su Despacho, informo que mi representada y la suscrita recibiremos notificaciones en la Secretaría de su Despacho o en mis oficinas situadas en la Carrera 13 N° 119-95, Of. 104 de esta ciudad de Bogotá.

Señor Superintendente,

ELIANA ISAURA MORENO BOHORQUEZ
C.C. 51'975.664 de Bogotá
T.P.A. 83823 del C.S. de la J.

10.

PROCAPS

PODER ESPECIAL

Yo, **WALTER TANAKA TATEKAWA,** mayor de edad y vecino de la ciudad de Barranquilla (Colombia), identificado como aparece al pie de mi firma, quien en el presente documento actúa en calidad de Representante Legal de la sociedad **PROCAPS S.A.** sociedad legalmente constituida y domiciliada en la ciudad de Barranquilla (Colombia), por el presente documento otorgo a la doctora **ELIANA ISAURA MORENO BOHORQUEZ**, abogada en ejercicio, identificada como aparece al pie de su firma, con tarjeta profesional No. 83823 **del C.S. de la J.**, poder especial, amplio y suficiente, para formular oposiciones u observaciones a la solicitud de patente No. **07.009.315,** titulada **"FORMULACIONES PARA ALMACENAR PROTEÍNAS"**, cuyo titular es **WYETH,** publicada en la gaceta No. 581 del 31 de Octubre de 2007.

Nuestro apoderado queda facultado, de manera expresa, para realizar todo tipo de actos necesarios para llevar a cabo el trámite de la oposición, incluyendo, sin limitación, preparar y presentar solicitudes, hacer declaraciones, pagar tasas, contribuciones e impuestos, recabar los títulos o certificados. Asimismo quedan autorizados para impugnar administrativa y judicialmente todo lo que fuere resuelto en el expediente a que se hace expresa referencia en el presente poder, con todas las facultades generales y específicas que fuera requerido para ella.

De la misma manera, además de las facultades que le confiere la naturaleza del presente mandato, se encuentran facultados para recibir, desistir, transigir, sustituir, y reasumir el presente poder, incoar acciones, interponer recursos y demás facultades conferidas por la ley.

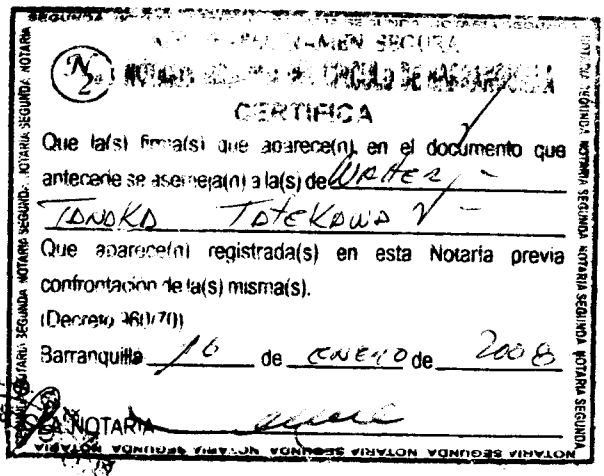
Dado y Firmado en Barranquilla (Colombia) a los 15 días del mes de enero de 2008.

PROCAPS S.A.
WALTER TANAKA TATEKAWA
C.C. N° 16.251.923 de Palmira
Representante Legal

Acepto:

ELIANA ISAURA MORENO BOHORQUEZ
C.C. 51'975.664 de Bogotá
T.P.A. 83823 del C.S. de la J.

DILIGENCIA DE PRESENTACIÓN
La Notaria Dieciocho del Círculo de Bogotá, D.C., hace constar que el presente documento fue presentado personalmente por
quien exhibió la C.C.
De y Tarjeta Profesional
No. C.S.J., y declaró que la firma y la huella que aparecen en el presente documento son suyas.
BOGOTÁ D.C. 28 ENE. 2008
FIRMA
Victoria Bernal Trujillo
NOTARIA
HUELLA DEL INDICE DERECHO



EXAMEN SECURA

CERTIFICA

Que la(s) firma(s) que aparece(n) en el documento que antecede se asemeja(n) a la(s) de WALTER TONOKO TOTEKOWA V.

Que aparece(n) registrada(s) en esta Notaria previa confrontacion de la(s) misma(s).

(Decreto 960/70)

Barranquilla 16 de ENERO de 2008

LA NOTARIA

NOTARIA SEGUNDA NOTARIA SEGUNDA NOTARIA SEGUNDA NOTARIA SEGUNDA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 08 - 7,663
FECHA : ENERO 29 DE 2008

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 07-009315- -00002-0000

Fecha: 2008-01-29 13:41:46 Dep: 2020 NUECREACIONE
Tra: 11 PATENTEIYII Eve: 378 FASENACIONALI
Act: 400 OPOSIOBSERVTER Folios: 129

***** C O N S I G N A C I O N *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
LAB. PROCAPS	CONSIGNACION	BANCO DE BOGOTA	062754387	534245	29/01/2008	30,000.00
LAB. PROCAPS	CONSIGNACION	BANCO POPULAR	050-00110-6	67792279	12/12/2007	404,000.00
LAB. PROCAPS	CONSIGNACION	BANCO POPULAR	050-00110-6	67792283	12/12/2007	404,000.00

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

CANT.	RENTISTICO	CONCEPTO		TOTAL CONCEPTO
1	50005-01-04 PRESENTACION DE OBSERVACIONES	12 MARCAS Y LEMAS COMERCIALES		266,000.00
		TOTAL :	$	266,000.00

SON: DOSCIENTOS SESENTA Y SEIS MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

03-07001890 024802 PROCAPS S.A.
Barranquilla, 16 de Enero de 2008 Hr:14:57:12

No. 11692896

CAMARA DE COMERCIO DE BARRANQUILLA

********** Pag. 1
C A M A R A D E C O M E R C I O
D E B A R R A N Q U I L L A
09**********

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.
PROCAPS S.A.----------
NIT: 890.106.527-5.

EL SUSCRITO SECRETARIO DE LA CAMARA DE COMERCIO DE BARRANQUILLA, CON FUNDAMENTO EN LAS INSCRIPCIONES DEL REGISTRO MERCANTIL:

C E R T I F I C A

Que por Escritura Pública No. 1,190 del 22 de Julio de 1976, otorgada en la Notaria Tercera de Barranquilla, inscrito(as) en esta Cámara de Comercio, el 28 de Julio de 1976 bajo el No. 6,070 del libro respectivo, fue constituida la sociedad----------
limitada denominada "PRODUCTORA DE CAPSULAS DE GELATINAS LIMITADA "PROCAPS LIMITADA".----------

C E R T I F I C A

Que por Escritura Pública No. 1,307 del 28 de Junio de 1979, otorgada en la Notaria Tercera de Barranquilla, inscrito(as) en esta Cámara de Comercio, el 23 de Julio de 1979 bajo el No. 10,039 del libro respectivo, la sociedad antes mencionada----------
se transformo en sociedad anonima bajo la denominacion social de "PRODUCTORA DE CAPSULAS DE GELATINA S.A." PROCAPS S.A.".----------

C E R T I F I C A

Que por Escritura Pública No. 3,810 del 31 de Dic/bre de 1980, otorgada en la Notaria Primera de Barranquilla, inscrito(as) en esta Cámara de Comercio, el 30 de Enero de 1981 bajo el No. 12,705 del libro respectivo, la sociedad antes mencionada----------
se transformo en sociedad de responsabilidad limitada bajo al razon social de "PRODUCTORA DE CAPSULAS DE GELATINA LIMITADA "PROCAPS LI - MITADA".----------

C E R T I F I C A

Que por Escritura Pública No. 3,755 del 10 de Nov/bre de 1983, otorgada en la Notaria Unica de Soledad, inscrito(as) en esta Cámara de Comercio, el 22 de Nov/bre de 1983 bajo el No. 17,922 del libro respectivo, la sociedad antes mencionada----------
se transformo en sociedad anonima bajo la denominacion social de "PRODUCTORA DE CAPSULAS DE GELATINA S.A. "PROCAPS S.A.".----------

C E R T I F I C A

Que por Escritura Pública No. 3,615 del 06 de Dic/bre de 1996, otorgada en la Notaria 2a. de Barranquilla, inscrito(as) en esta Cámara de Comercio, el 20 de Dic/bre de 1996 bajo el No. 67,218 del

********** C O N T I N U A **********

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.
PROCAPS S.A.----------
NIT: 890.106.527-5.

libro respectivo, la sociedad antes mencionada----------
cambio de razon social, por la denominacion LABORATORIOS PROCAPS S.A
. ----------

C E R T I F I C A

Que por Escritura Pública No. 3,775 del 18 de Dic/bre de 1996, otorgada en la Notaria 2a. de Barranquilla, inscrito(as) en esta Cámara de Comercio, el 23 de Enero de 1997 bajo el No. 67,704 del libro respectivo, la sociedad antes mencionada----------
cambio de razon social, por la denominacion PROCAPS S.A. ----------

C E R T I F I C A

Que dicha sociedad ha sido reformada por las siguientes escrituras y/o documentos privados:

Numero	aaaa/mm/dd	Notaria	No. Insc o Reg	aaaa/mm/dd
39	1979/01/18	Notaria 3a. de Barranquilla.	9,452	1979/01/24
992	1981/04/30	Notaria 1a. de Barranquilla.	13,112	1981/05/06
1,464	1982/08/27	Notaria Unica de Soledad	15,533	1982/09/08
2,744	1983/08/18	Notaria Unica de Soledad	17,416	1983/08/26
3,826	1984/07/16	Notaria Unica de Soledad	19,554	1984/07/27
3,835	1985/08/26	Notaria Unica de Soledad	22,230	1985/09/04
2,260	1986/09/10	Notaria Unica de Soledad.	24,995	1986/09/15
1,849	1989/07/11	Notaria 2a. de Barranquilla.	33,909	1989/07/13
444	1990/02/21	Notaria 2a. de Barranquilla.	36,101	1990/03/16
3,090	1991/12/17	Notaria 2a. de Barranquilla	43,993	1992/01/28
269	1993/02/01	Notaria 2a. de Barranquilla	48,428	1993/02/16
2,342	1993/07/28	Notaria 2a. de Barranquilla	50,582	1993/08/10
3,652	1993/11/09	Notaria 2a. de Barranquilla	51,798	1993/11/22
3,615	1996/12/06	Notaria 2a. de Barranquilla	67,218	1996/12/20
89	1997/01/16	Notaria 2a. de Barranquilla	67,704	1997/01/23
2,070	1997/07/17	Notaria 2a. de Barranquilla	70,627	1997/07/31
2,281	1997/08/04	Notaria 2a. de Barranquilla	70,813	1997/08/14
765	1999/04/22	Notaria 3a. de Barranquilla	80,628	1999/04/23
459	2001/02/21	Notaria 2a. de Barranquilla	91,502	2001/02/23
2,608	2001/10/05	Notaria 2a. de Barranquilla	95,771	2001/11/08
1,730	2004/07/29	Notaria 2. de Barranquilla	112,999	2004/08/26
416	2005/03/03	Notaria 2. de Barranquilla	116,364	2005/03/10
1,613	2005/07/28	Notaria 2. de Barranquilla	119,322	2005/08/18

C E R T I F I C A

Que de acuerdo con la(s) escritura(s) arriba citada(s), la sociedad se rige por las siguientes disposiciones:
DENOMINACION O RAZON SOCIAL:
PROCAPS S.A.----------
SIGLA: PROCAPS.
DOMICILIO PRINCIPAL: Barranquilla.
NIT No: 890.106.527-5.
MATRICULA MERCANTIL: 24,802.

C E R T I F I C A

********** C O N T I N U A **********

03-07001890 024802 PROCAPS S.A.
Barranquilla, 16 de Enero de 2008 Hr:14:57:12

No. 11692897

CAMARA DE COMERCIO DE BARRANQUILLA

C A M A R A D E C O M E R C I O
D E B A R R A N Q U I L L A
09**********

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.
PROCAPS S.A.----------
NIT: 890.106.527-5.

Direccion para notificaciones judiciales:
CL 80 No 78 B - 201.
De la ciudad de Barranquilla.

C E R T I F I C A

DURACION: El término de duración de la sociedad se fijó hasta el 18 de Agosto de 2073.

C E R T I F I C A

OBJETO SOCIAL: El objeto principal de la sociedad sera: a)-La fabricacion de capsulas y otros productos similares a partir de materiales tales como gelatinas u otros de distinta naturaleza, que puedan ser utilizados como envases o recipientes de productos y sustancias quimicas, farmaceuticas y cosmeticas, para uso y consumo humano y veterinario; b)-La investigacion, desarrollo y fabricacion de toda clase de productos y sustancias quimicas, farmaceuticas y cosmeticas para uso y consumo humano, animal y vegetal; c)-La compra y venta de materias primas, insumos y productos intermedios necesarios para los procesos de fabricacion de sustancias quimicas, farmaceuticas,alimenticias y cosmeticas, asi como la comercializacion interna y externa de los productos terminados, d) La compra, venta, importacion, exportacion y distribucion de toda clase de elementos instrumentales y equipos medicos,quirurgicos,odontologicos,y hospitalarios para la salud humana y animal. e)La compra,venta,exportacion y disdistribucion de dulces, confites, galletas y todo tipo de golosinas o alimentos para el consumo humano. f) Actuar como representante o agente de personas naturales y juridicas, nacionacionales y extranjeras en la realizacion de operaciones o transacciones relacionadas con la industria farmaceutica y en especial con cualquiera de las actividades descritas en los literales a), b), c). d) y e) antes descritos; g) Prestar los servicios de asesoria y consultoria en las areas administrativas, de mercadeo, analisis clinico, asistencia tecnica, investigacion, costos y produccion, exportaciones, importaciones, ventas, relacionadas con iguales o similares actividades a las que constituyen el objeto de PROCAPS S.A., asi como los de publicidad y promocion de productos quimicos, cosmeticos y farmaceuticos para uso humano, animal o industrial; h)-Adquirir, operar, administrar y explotar, a cualquier titulo, empresa o establecimientos dedicados a la investigacion, desarrollo, fabricacion o comercializacion de productos quimicos, farmaceuticos, hospitalarios o cosmeticos. En desarrollo de su objeto principal, la sociedad podra tomar interes social como accionista,

********** C O N T I N U A **********

socio o fundador de empresas que tengan igual o similar objeto, comprar, vender, importar y exportar maquinarias, insumos y repuestos requeridos para el desarrollo de sus actividades; tomar dinero en prestamo, financiar y garantizar las operaciones comerciales que celebre con terceros relativas a su objeto; celebrar y ejecutar todo tipo de actos y contratos necesarios para desarrollar sus operaciones; girar, aceptar, otorgar y protestar todo tipo de titulos valores e instrumentos negociables; comprar, vender y constituir gravamenes sobre bienes muebles e inmuebles; designar representantes o apoderados en el pais y en el exterior y, en general, realizar cualquier acto o negocio que sea necesario o conveniente para el desarrollo de su objeto social y para la mejor proteccion de sus intereses corporativos o la de sus socios. La sociedad podra garantizar el cumplimiento de obligaciones adquiridas por terceros o por sus socios, para lo cual podra inclusive constituir gravamenes reales sobre sus bienes de cualquier naturaleza.----------

C E R T I F I C A

CAPITAL	Nro Acciones	Valor Acción
Autorizado		
$**********2,000,000,000	**********2,000,000	**********1,000
Suscrito		
$**********1,224,500,000	**********1,224,500	**********1,000
Pagado		
$**********1,229,071,000	**********1,229,071	**********1,000

C E R T I F I C A

ADMINISTRACION: La direccion y administracion de la sociedad corresponderа a la Asamblea General de Accionistas, a la Junta Directiva, al Presidente, al Vicepresidente y a un Gerente General. Corresponde a la Junta Directiva las siguientes funciones y atribuciones entre otras: Autorizar cualquier acto o contrato cuya cuantia sea superior al equivalente en pesos Colombianos a doscientos cincuenta mil dolares de los Estados Unidos de America (US$250.000), liquidados a la tasa de cambio representativa del mercado vigente el dia en que se surta la transacion. Cuando se trate de operaciones en virtud de las cuales la sociedad garantice obligaciones de terceros o de los socios, se requerira que la operacion sea aprobada con el voto de por lo menos 2/3 partes de los integrantes de la Junta Directiva.
La sociedad tendra un Presidente , quien ejercera la representacion legal de la sociedad y tendra las siguientes atribuciones entre otras: Ejecutar las decisiones de la Asamblea General de Accionistas y de la Junta Directiva. Ejercer la Representacion Legal de la sociedad en todos los actos y contratos que se requieran para el desarrollo del objeto social, de conformidad con lo previsto en las leyes y en los presentes estatutos. Constituir apoderados judiciales y extrajudiciales, recibir notificaciones, absolver interrogatorios de parte, confesar obligaciones de la sociedad, transigir, disistir y allanar a la sociedad en cualquier asunto judicial. Aprobar y celebrar en nombre de la socieda todo acto o contrato cuya cuantia sea igual o inferior al equivalente

********** C O N T I N U A **********

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.
PROCAPS S.A.----------
NIT: 890.106.527-5.

en pesos colombianos a doscientos cincuenta mil dolares de los Estados Unidos de America (US$250.000), liquidados a la tasa de cambio representativa del mercado vigente el dia en que se surta la transacion o celebracion del acto. Las demas funciones que le correspondan como Representante Legal por disposicion de la ley, de estos estatutos o de la Junta Directiva. El Presidente tendra un suplente, designado por la Junta Directiva, quien reemplazara al Presidente en sus faltas temporales, absolutas o meramente accidentales, con entidades facultades y sin que sea necesario para ello acreditar la ausencia del principal. La sociedad tendra un Vicepresidente designado por la Junta Directiva, quien tambien ejercera la representacion legal de la sociedad, pudiendo ejercerla conjunta o separadamente con el Presidente. El vicepresidente tendra las siguentes facultades entre otras. Ejecutar dentro de su competencia las decisiones de la Asamblea General de Accionistas, de la Junta Directiva y del Presidente. Ejercer la representacion legal de la sociedad en todos los actos y contratos que se requieran para el desarrollo del objeto social, de conformidad con lo previsto en las leyes y en los presentes estatutos. Constituir apoderados judiciales y extrajudiciales, recibir notificaciones, absolver interrogatorios de parte, confesar obligaciones de la sociedad, transigir, desistir y allanar a la sociedad en cualquier asunto judicial. Aprobar y celebrar en nombre de la sociedad todo acto o contrato cuya cuantia sea igual o inferior al equivalente en pesos colombianos a ciento cincuenta mil dolares de los Estados Unidos de America (US$150.000), liquidados a la tasa de cambio representativa del mercado vigente el dia en que se surta la aprobacion o celebracion del acto, siempre que no se trate de enajenar bienes inmuebles o cualquier otro activo fijo de la sociedad, inclusive de naturaleza muebles, acciones, cuotas o partes de interes inversiones y todo tipo de derechos de la sociedad, o de constituir gravamenes sobre cualquiera de los anteriores, facultades que radican exclusiva y privativamente en cabeza del Presidente. Las demas funciones que le corresponda como Representante legal por disposicion de la ley, de estos estatutos o de la Junta Directiva. El Vicepresidente tendra (1) suplente, designado por la Junra Directiva, quien lo reemplazara conjunta o separadamente en sus faltas temporales, absolutas o meramente accidentales, con identicas facultades y sin que sea necesario para ello acreditar la ausencia del principal. La sociedad tendra un (1) Gerente General, con su respectivo Suplente, designados por la Junta Directiva. El Suplente reemplazara al Gerente General, en sus faltas temporales, absolutas o meramente accidentales, quien junto con el gerente general, llevaran la representacion legal de la sociedad, con identicas facultades, pudiendolas ejercer conjunta o separadamente con el Presidente y Vicepresidente, con identicas facultades

********** C O N T I N U A **********

que el Gerente General y sin que sea necesario para ello acreditar la ausencia del titular. El Gerente General tendra las siguientes facultades entre otras: Ejercer la representacion legal de la sociedad, en todos los actos y contratos que se requieran para el desarrollo del objeto social, de conformidad con lo previsto en las leyes y en los presentes estatutos. Aprobar y celebrar en nombre de la sociedad todo acto o contrato cuya cuantia sea igual o inferior al equivalente en pesos Colombianos a Cincuenta Mil dolares de los Estados Unidos de America (US$50.000), liquidados a la tasa de cambio representativa del mercado vigente el dia en que se surta la aprobacion o celebracion del acto, siempre que no se trate de enajenar a cualquier titulo bienes inmuebles o cualquier otro activo fijo de la sociedad, inclusive de naturaleza mueble, acciones, cuotas o partes de interes, inversiones y todo tipo de derechos de la sociedad o de constituir gravamenes sobre cualquiera de las anteriores facultades que radican exclusiva y privativamente en cabeza del Presidente. ----------

C E R T I F I C A

Que según Acta No. 116 del 27 de Junio de 1997 correspondiente a la Asamblea de Accionistas en Barranquilla, de la sociedad:
PROCAPS S.A.----------
cuya parte pertinente se inscribió en esta Cámara de Comercio, el 15 de Agosto de 1997 bajo el No. 70,838 del libro respectivo, y según Acta No. 127 del 22 de Marzo de 2000 correspondiente a la Asamblea de Accionistas en Barranquilla, de la sociedad:
PROCAPS S.A.----------
cuya parte pertinente se inscribió en esta Cámara de Comercio, el 29 de Mayo de 2000 bajo el No. 87,250 del libro respectivo, fueron hechos los siguientes nombramientos:

CLASE: JUNTA DIRECTIVA

Principales

1. Minski Gontovnik Ruben	CC.*******7,463,982
2. Minski Gontovnik Meyer	CC.*******7,461,324
3. Minski Gontovnik Jose	CC.*******8,671,834

Suplentes

1. Minski Zilberblum Elliot	CC.******72,219,541
2. Minski Deitel Daniel	.****************
3. Minski Sharon	PA.*990,212,082,805

C E R T I F I C A

Que según Acta No. 116 del 27 de Junio de 1997 correspondiente a la Asamblea de Accionistas en Barranquilla, de la sociedad: PROCAPS S.A. cuya parte pertinente se inscribió en esta Cámara de Comercio, el 15 de Agosto de 1997 bajo el No. 70,838 del libro respectivo, fueron hechos los siguientes nombramientos:

Cargo/Nombre	Identificación
Presidente.	

********** C O N T I N U A **********

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.
PROCAPS S.A.---
NIT: 890.106.527-5.

Minski Gontovnik Ruben	CC.*******7,463,982
Suplente del Presidente	
Minski Minski Abraham	CC.*********802,575
Vicepresidente	
Minski Gontovnik Meyer	CC.*******7,461,324
Suplente del vicepresidente	
Minski Gontovnik Jose	CC.*******8,671,834

C E R T I F I C A

Que según Acta No. 11 del 01 de Febrero de 2001 correspondiente a la Junta Directiva en Barranquilla, de la sociedad: PROCAPS S.A. cuya parte pertinente se inscribió en esta Cámara de Comercio, el 12 de Febrero de 2001 bajo el No. 91,273 del libro respectivo, fueron hechos los siguientes nombramientos:

Cargo/Nombre	Identificación
Gerente General.	
Uribe Ochoa Guillermo Luis	CC.******70,073,754

C E R T I F I C A

Que según Acta No. 406 del 02 de Sep/bre de 2004 correspondiente a la Junta Directiva en Barranquilla, de la sociedad: PROCAPS S.A. cuya parte pertinente se inscribió en esta Cámara de Comercio, el 09 de Sep/bre de 2004 bajo el No. 113,263 del libro respectivo, fueron hechos los siguientes nombramientos:

Cargo/Nombre	Identificación
Suplente del Gerente.	
Tanaka Tatekawa Walter	CC.******16,251,923

C E R T I F I C A

Que según Acta No. 124 del 29 de Marzo de 1999 correspondiente a la Asamblea de Accionistas en Barranquilla, de la sociedad: PROCAPS S.A. cuya parte pertinente se inscribió en esta Cámara de Comercio, el 04 de Agosto de 1999 bajo el No. 82,226 del libro respectivo, fueron hechos los siguientes nombramientos:

Cargo/Nombre	Identificación
Revisor Fiscal Ppal.	
Vargas Pertuz Ana Isabel	CC.******32,696,645

C E R T I F I C A

Que según Acta No. 127 del 22 de Marzo de 2000 correspondiente a la Asamblea de Accionistas en Barranquilla, de la sociedad: PROCAPS

********** C O N T I N U A **********

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.

PROCAPS S.A.----------

NIT: 890.106.527-5.

S.A. cuya parte pertinente se inscribió en esta Cámara de Comercio, el 29 de Mayo de 2000 bajo el No. 87,250 del libro respectivo, fueron hechos los siguientes nombramientos:

Cargo/Nombre	Identificación
Suplente del Revisor Fiscal.	
Lozada Villareal Milena	CC.******32,748,111

C E R T I F I C A

Que por Escritura Pública No. 2,784 del 07 de Dic/bre de 2007, otorgada en la Notaria 2 a. de Barranquilla cuya parte pertinente se inscribió en esta Cámara de Comercio, el 20 de Dic/bre de 2007 bajo el No. 3,492 del libro respectivo,---------- y las inscripciones 3.492-1, 3.493, 3.494, 3.495, 3.496, 3.497, ---- 3.498, 3.499, 3.500, 3.501, 3.502, 3.503, 3.504, 3.505, 3.506, ----- 3.507, Consta que RUBEN MINSKI GONTOVNIK, C.C. No. 7.463.982, ------ obrando como Presidente y Representante Legal de la sociedad C.I. NATURMEGA S.A., que obrando en su condicion de Presidente y ------- Representante Legaql de la sociedad PROCAPS S.A., el presidente ---- debidamente facultado por los Estatutos Sociales y por el Maximo Organo Social, otorga los siguientes poderes: Otorgar poder -------- general, amplio y suficiente a los doctores JOSEFINA DEL CARMEN ---- MIELES BUELVAS CC.No.33.138.497; a MARCELA CARVAJALINO PAGANO ------ CC.No.22.579.748; CRISTINA VIOLI FRANCESCHINI CC.No.22.463.716, con las facultades que se transcriben a continuacion: a.- Para que ----- representen a la sociedad PROCAPS S.A, ante todo tipo de ---------- autoridades administrativas y jurisdiccionales, en asuntos de ------ naturaleza civil, laboral, comercial, penal, aduanera, cambiaria, tributaria, policiva, administrativa y fiscal. b.- Contestar y ----- presentar demandas, requerimientos, recursos, pedir y aportar ------ pruebas, recibir, notificarse, ejercer la accion de tutela, -------- tramitar incidente de desacato, comparecer en los procesos que ----- cursen contra la sociedad, allanarse en nombre de la sociedad en los asuntos que sean necesarios, transigir, desistir, sustituir, renunciar y reasumir el poder. c.- La facultad expresa de ---------- conciliar, por tanto pueden llegar a acuerdos conciliatorios, ------ transaccionales, judiciales o extrajudiciales o extrajudiciales, cualquiera que sea su cuantia, para lo cual las apoderadas deben aportar la autorizacion simple y escrita del Representante legal de la sociedad. d.- Podran presentar solicitudes de devolucion y/o compensacion de impuestos, ventas y rentas, recibir los pagos por las devoluciones y adelantar todo tramite o proceso administrativo ante la DIAN, de tal forma que en ningun momento quede sin --------- representacion legal de la sociedad. PODERES ESPECIALES: a.- Se ---- confiere poder especial amplio y suficiente a las personas que se determinan seguidamente, que conformen el grupo A y B, para, para que conjuntamente suscriban contratos de suministros de mercancias y servicios con entidades oficiales, hasta la suma de OCHOCIENTOS MIL DOLARES DE LOS ESTADOS UNIDOS DE AMERICA (US800.000.00), ------- liquidados a la tasa representativa del mercado vigente en el dia que se realice la respectiva transaccion. Grupo A. WALTER NANAKA TATEKAWA CC.No.16.251.923, CLAUDIA TANGARIFE CASTILLO ---------- CC.No.41.660.293, CARMEN ALICIA FLOREZ CC.No.22.434.044, IVAN ------

********** C O N T I N U A **********

03-07001890 024802 PROCAPS S.A.
Barranquilla, 16 de Enero de 2008 Hr:14:57:12

No. 11692900

CAMARA DE COMERCIO DE BARRANQUILLA

********** Pag. 5
CAMARA DE COMERCIO
DE BARRANQUILLA
09**********

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.
PROCAPS S.A.----------
NIT: 890.106.527-5.

VILLAREAL CAMACHO CC.No.91.201.749, CARMEN ALICIA HERRERA ---------- DIAZGRANADOS CC.No.22.377.540, DAVID ANTONIO CHARRIS GIRALDO ------- CC.No.19.341.274, RICARDO DORADO HURTADO CC.No.79.715.772, JAIME FORERO LLINAS CC.No.19.300.593. b.- Se confiere poder especial ----- amplio y suficiente al senor WILLIAM ALBERTO BARROS CERRA ---------- CC.No.8.715.941, y al senor ANTONIO VARELA DIAZ CC.No.72.004.699, para que en nombre y representacion de PROCAPS S.A., suscriban ----- declaraciones cambiarias, aduaneras y tributarias a que este ------- obligada la sociedad, en el giro normal de sus negocios y ---------- operaciones sociales, por tanto quedan facultados para firmarlas, como tambien los demas documentos relacionados con ellas, ---------- notificarse, aportar y solicitar las pruebas que sean necesarias. c.-Otorgar poder especial amplio y suficiente al senor MARLON ----- TATIS PAREDES CC.No.72.176.874 y al senor WILLIAM ALBERTO BARROS CERRA CC.No.8.715.941, para que en nombre y representacion de ------ PROCANPS S.A., suscriban las declaraciones, reportes y cualquier documento relacionado con los tramites o registros de Comercio ----- Exterior y o cualquier entidad del Estado que haga sus veces: d) Otorgar poder especial amplio y suficiente a la doctora CARMEN ----- ALICIA HERRERA DIAZGRANADOS, CC.No.22.377.540 y al doctor WALTER TANAKA TATEKAWA, CC.No.16.251.923, para que en nombre y ---------- representacion de PROCAPS S.A.; endosen los titulos valores de la sociedad hasta por la suma de UN MIL MILLONES DE PESOS ---------- (1.000.000.000.00) M.L. CUARTO: EI representante legal de la ------- sociedad PROCAPS S.A.; doctor RUBEN MINSKI GONTOVNIK, manifiesta que la presente reforma fue aprobada por la Asamblea General ------- Extraordinaria de Accionista de PROCAPS S.A., con observancia de las disposiciones legales y estatutarias.----------

C E R T I F I C A

Que en esta Cámara de Comercio no aparecen inscripciones posteriores de documentos referentes a reforma, disolución, liquidación o nombramientos de representantes legales de la expresada sociedad.

C E R T I F I C A

Que su última Renovación fue el: 20 de Marzo de 2007.

La información sobre embargos de establecimiento se suministra en Certificados de Matrícula, la de contratos sujetos a registro, en Certificados Especiales.

CERTIFICADO DE EXISTENCIA Y REPRESENTACION LEGAL O DE INSCRIPCION DE DOCUMENTOS.

PROCAPS S.A.----------

NIT: 890.106.527-5.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 39/395, 39/00, C07K 1/00 — A1

(11) International Publication Number: **WO 98/56418**

(43) International Publication Date: 17 December 1998 (17.12.98)

(21) International Application Number: PCT/US98/12209

(22) International Filing Date: 12 June 1998 (12.06.98)

(30) Priority Data:
08/874,897 13 June 1997 (13.06.97) US

(71) Applicant: GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080–4990 (US).

(72) Inventors: LAM, Xanthe, M.; 280 Denslowe Drive, San Francisco, CA 94132 (US). OESWEIN, James, Q.; P.O. Box 13, Moss Beach, CA 94038 (US). ONGPIPATTANAKUL, Boonsri; 55–35 Charlerm Khet 2 Road, Pomprah, Bangkok 10100 (TH). SHAHROKH, Zahra; 24 Sotelo Avenue, San Francisco, CA 94116 (US). WANG, Sharon, X.; 3187 Greenoak Court, San Mateo, CA 94403 (US). WEISSBURG, Robert, P.; 1686 Oxford Street, Berkeley, CA 94709 (US). WONG, Rita, L.; 2381 Ticonderoga, San Mateo, CA 94402 (US).

(74) Agents: LEE, Wendy, M. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080–4990 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: STABILIZED ANTIBODY FORMULATION

(57) Abstract

A stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody not subjected to prior lyophilization, a buffer maintaining the pH in the range from about 4.5 to about 6.0, a surfactant and a polyol is described, along with uses for such a formulation.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

STABILIZED ANTIBODY FORMULATION

Background of the Invention

Field of the Invention

This invention is directed to a stable aqueous pharmaceutical formulation comprising an antibody.

Description of Related Disclosures

In the past ten years, advances in biotechnology have made it possible to produce a variety of proteins for pharmaceutical applications using recombinant DNA techniques. Because proteins are larger and more complex than traditional organic and inorganic drugs (*i.e.* possessing multiple functional groups in addition to complex three-dimensional structures), the formulation of such proteins poses special problems. For a protein to remain biologically active, a formulation must preserve intact the conformational integrity of at least a core sequence of the protein's amino acids while at the same time protecting the protein's multiple functional groups from degradation. Degradation pathways for proteins can involve chemical instability (*i.e.* any process which involves modification of the protein by bond formation or cleavage resulting in a new chemical entity) or physical instability (*i.e.* changes in the higher order structure of the protein). Chemical instability can result from deamidation, racemization, hydrolysis, oxidation, beta elimination or disulfide exchange. Physical instability can result from denaturation, aggregation, precipitation or adsorption, for example. The three most common protein degradation pathways are protein aggregation, deamidation and oxidation. Cleland *et al. Critical Reviews in Therapeutic Drug Carrier Systems* 10(4): 307-377 (1993).

Included in the proteins used for pharmaceutical applications are antibodies. An example of an antibody useful for therapy is an antibody which binds to the CD18 antigen. CD18 is the common β subunit of three heterodimeric membrane integrins restricted to leukocytes that mediate trafficking and adhesion to the vascular endothelium, particularly at sites of inflammation (for reviews see Hynes, R.O. *Cell*, 69:11-25 (1992); Stoolman, *Cell*, 56:907-910 (1989); Jutila *et al. Transplantation* 48(5):727-731 (1989); Springer, T.A., *Nature* 346:425-434 (1990); and Albelda and Buck, *FASEB J.* 4:2868-2880 (1990)). The heterodimer containing CD18 and CD11b (also called MAC-1) is found primarily on neutrophils, monocytes, and some lymphocytes whose normal interaction with ICAM-1 on vascular endothelium mediates adhesion and "rolling" of cells along the vasculature. In severe hemorrhagic trauma with concurrent decrease in cardiac output and ischemia, early (within 30 min) neutrophil activation (in response to released cytokines) and up-regulation of MAC-1 increases neutrophil "stickiness". This precedes extravasation and release of proteases and superoxides that ultimately lead to further tissue damage and increased vascular permeability (Hernandez *et al.*, *Am. J. Physiol.*, 253(3 Pt 2): H699-H703 (1987)). Reperfusion following resuscitation exacerbates the edema and necrosis, and leads to multi-organ failure and death. Early treatment with monoclonal antibodies to CD18 in a partially-severed, ischemic rabbit ear trauma model alleviated tissue necrosis following reattachment (Vedder *et al.*, *J. Clin. Invest.* 81:939-944 (1988)). A humanized antibody showed efficacy in reducing multi-organ damage and death in a rhesus monkey model of decreased cardiac output (created by depletion of 2/3 of blood volume for ~ 2 hours (Mileski *et al.*, *Surgery*, 108(2):206-

212 (1990)). These studies point to the therapeutic potential of anti-CD18 antibodies for acute treatment of hemorrhagic shock.

Another antigen of interest for targeting with antibodies is the CD20 antigen, also known as "Bp35". CD20 is a human B cell marker which is expressed during early pre-B cell development and remains until plasma cell differentiation. The CD20 molecule may regulate a step in the activation process which is required for cell cycle initiation and differentiation and is usually expressed at very high levels on neoplastic B cells. Thus, the CD20 surface antigen can be targeted for treating B cell lymphomas. US Patent 5,736,137 issued April 7, 1998 describes the chimeric antibody "C2B8" which binds the CD20 antigen and its use to treat B cell lymphoma.

There is a need in the art for a stable aqueous pharmaceutical formulation comprising an antibody, such as an anti-CD18 or anti-CD20 antibody, which is suitable for therapeutic use.

Summary of the Invention

Accordingly, the invention provides a stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody not subjected to prior lyophilization, a buffer maintaining the pH in the range from about 4.5 to about 6.0, a surfactant and a polyol. Preferably the formulation is stable at a temperature of about 2-8°C for at least one year, and/or is stable at a temperature of about 30°C for at least one month and/or is stable following freezing and thawing of the formulation.

The invention also relates to an article of manufacture comprising a container holding a stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody not subjected to prior lyophilization, a buffer maintaining the pH in the range from about 4.5 to about 6.0, a surfactant and a polyol.

In yet a further aspect, the invention relates to a method for stabilizing an antibody in an aqueous pharmaceutical formulation by combining a therapeutically effective amount of an antibody not subjected to prior lyophilization, a buffer maintaining the pH in the range from about 4.5 to about 6.0, a surfactant and a polyol.

In a still further aspect, the invention concerns a method of treating a mammal comprising administering a therapeutically effective amount of the aqueous pharmaceutical formulation disclosed herein to a mammal, wherein the mammal has a disorder requiring treatment with the antibody in the formulation. Where the antibody binds CD18, examples of disorders to be treated include hemorrhagic shock, thermal injury (such as that resulting from burns), stroke (including ischemic and hemorrhagic stroke) and myocardial infarction. For an anti-IL8 antibody, disorders to be treated include inflammatory disorders such as adult respiratory distress syndrome (ARDS), hypovolemic shock, ulcerative colitis, and rheumatoid arthritis. Where the antibody binds CD20, disorders to be treated include B cell lymphomas.

These and further aspects of the invention will be apparent to those skilled in the art.

Brief Description of the Drawings

Figures 1A and 1B depict the amino acid sequence of rhuMAb CD18 heavy chain (Figure 1A; SEQ ID NO:1) and light chain (Figure 1B; SEQ ID NO:2). The sequence in italics in Figure 1A (SEQ ID NO:3) is that of the leucine zipper.

Figures 2A and 2B are hydroflex plots of rhuMAb CD18 heavy chain (Figure 2A) and light chain (Figure 2B). Kyte-Doolittle hydrophobicity calculations averaged with a window of 6 amino acids were made on the protein sequence. Flexibility values were estimated from the product of the hydrophobicity of each residue and its side chain volume (again averaged over a window of 6 residues). Asn-Gly and Asn-Ser motifs in flexible regions are more likely to deamidate than those in more rigid structures. The CDRs are also shown. Most of the heavy chain methionines and heavy chain Asn 84 are near the CDR's.

Figures 3A and 3B show the effect of storage temperature on light scattering in different rhuMAb CD18 formulations after 5 weeks storage at the designated temperatures as measured by average absorbance in the range of 340-360 nm (Figure 3A), or the ratio of A278 over A252 nm (Figure 3B). RhuMAb CD18 in formulation F3 is prone to the formation of insoluble aggregates.

Figure 4 shows the effect of storage temperature on protein concentration of rhuMAb CD18 formulations measured by absorbance at 278 nm corrected for vehicle absorbance at 320 nm.

Figures 5A and 5B depict the effect of storage temperature on the stability of rhuMAb CD18 formulation F2 (Figure 5A) and formulation F5 (Figure 5B) assayed by size exclusion chromatography (SEC). A smaller MW species (see arrows) appeared which was more pronounced at pH 6 (Figure 5B) compared to pH 5 (Figure 5A).

Figures 6A and 6B represent the effect of storage time and temperature on the stability of rhuMAb CD18 showing total peak area (Figure 6A), and % main peak (Figure 6B) for all formulations, assayed by SEC. No significant change in total peak area was noted. F1 and F5 maintained the lowest % main peak.

Figure 7 is an Arrhenius plot of the % main peak area from SEC of rhuMAb CD18 formulated in acetate with trehalose at pH 5 (F2). Activation Energy = 19 ± 6kcal/mole.

Figure 8 shows the effect of storage for 5 weeks at 40°C on different rhuMAb CD18 formulations, assayed by hydrophobic interaction chromatography (HIC). The early eluting peaks at 17.5 min and the shoulder at the leading edge of the main peak increased compared to controls at -70°C. F2 showed the least increase in both components (see Figure 9).

Figure 9 shows the effect of storage for 5 weeks at different temperatures on formulation F2, assayed by HIC. A pre-main peak became apparent compared to -70°C control.

Figure 10 depicts the effect of storage temperature on the stability of rhuMAb CD18 (5 week data) assayed by HIC showing total peak area recovered. A trend towards loss in area was noted in all formulations with increasing storage temperatures, except for F5 which started lower to begin with.

Figure 11 shows the effect of storage for 5 weeks at different temperatures on formulation F2, assayed by reverse phase-hydrophobic liquid chromatography (RP-HPLC). A small partially resolved pre-main peak component (see arrow) increased at higher temperatures, while its slightly earlier eluting neighbor remained unchanged.

Figure 12 shows the effect of storage for 5 weeks on the stability of rhuMAb CD18 assayed by RP-HPLC showing percentage of the main peak. F2 and F3 showed the highest % main peak after 5 weeks at 40°C.

Figure 13 is an Arrhenius plot based on the RP-HPLC % main peak for formulation F2. Activation energy (~20 kcal/mole) was only approximated from these data due to the very small κ for 5°C, it appears similar to that obtained with the SEC and ion-exchange HPLC (IEX) assays.

Figures 14A and 14B show the effect of storage temperature on the stability of rhuMAb CD18, assayed by IEX, on formulation F2 at pH 5 (Figure 14A) and F5 at pH 6 (Figure 14B). Two pre-main peaks increased with increasing time and temperature, more pronounced at pH 6. A hump from 22 to 28 minutes is an artifact due to an impurity washing off the column.

Figure 15 is an Arrhenius plot of the % main peak area from IEX of rhuMAb CD18 formulated in acetate with trehalose at pH 5 (F2). Activation Energy = 20± 10 kcal/mole.

Figure 16 shows the three dimensional structure of rhuMAb CD18, including positions of methionine residues. Met 65 and Met 83 are exposed, whereas others are buried in the structure and are expected to be less labile to oxidation.

Figure 17 shows fluorescence spectroscopy of rhuMAb CD18 formulations. The emission spectra of various formulations were obtained on an SLM8000 fluorimeter, using 280nm excitation wavelength. 500 µL samples were placed in quartz cuvettes and spectra were obtained with a 2nm bandwidth at 22°C. Samples were prepared at the different pHs by dilution of a concentrated stock to 0.1 mg/mL protein and analyses were made after 24 hr at 25°C. The antibody is conformationally stable above pH 3.

Figures 18A and 18B depict the effect of pH and protein concentration on the stability of rhuMAb CD18. Formulations in the pH range of 3 to 6 and concentration range of 0.5 to 25 mg/mL were placed on stability at 40°C and -70°C for 2 months. Analyses were made by IEX (Figure 18A) and SEC (Figure 18B). pH 5 was found to be the preferred pH, irrespective of protein concentration.

Figures 19A, 19B and 19C depict the kinetics of degradation of rhuMAb CD18 by SEC (Figure 19A), IEX (Figure 19B) and MAC-1 binding (Figure 19C) at various temperatures. Duplicate samples in 10mM Na acetate, 8% trehalose, 0.01% TWEEN 20™, pH 5 were prepared in 3 cc glass vials and placed on stability at the indicated temperatures. In Figure 19C, specific activity was the ratio of MAC-1 binding to total $F(ab')_2$ ELISA. Samples are stable at 5°C and 15°C up to 43 weeks and at 30°C up to 1 month. Analyses were made at the time points indicated.

Figure 20 depicts the structure of plasmid pS1130 used to produce rhuMAb CD18 of the example below.

Figures 21A and 21B depict the full nucleotide sequence (SEQ ID NO:9) and encoded amino acid sequences (SEQ ID No's:10 and 11, respectively) of the pS1130 expression cassette.

Figure 22 shows derivation of the 49A5 production cell line.

Figure 23 is a schematic of the fermentation process for rhuMAb CD18.

Figures 24A and 24B depict the effect of pH on the rate of aggregation (Figure 24A) and oxidation (Figure 24B) of 40 mg/mL rhuMAb CD20, 25 mM histidine, 0.02% polysorbate formulations at pH 5, 6.5 or 7.5 stored at 40°C.

Figure 25 depicts the effect of excess molar ratios of trehalose on the freeze-thaw induced aggregation of rhuMAb CD20 multidose formulations. Each formulation is composed of 40 mg/mL rhuMAb CD20, 20 mM acetate, 0 to 1000:1 molar ratio of trehalose, 0.9% benzyl alcohol and 0.02%

polysorbate 20, pH 5.0 The amount of trehalose in formulations 1-4 was follows: 1 = 0 moles trehalose: 1 mole of rhuMAb CD20 (0 mM trehalose); 2 = 250 moles trehalose: 1 mole rhuMAb CD20 (67 mM trehalose); 3 = 500 moles trehalose: 1 mole rhuMAb CD20 (134 mM trehalose); and 4 = 1000 moles trehalose: 1 mole rhuMAb CD20 (267 mM trehalose).

Figure 26 shows the effect of excess molar ratios of trehalose or sodium chloride on the clarity of rhuMAb CD20 multidose formulations stored at 40°C for up to four weeks. The composition of the formulations is 40 mg/mL rhuMAb CD20, 20 mM acetate, 0 to 1000:1 molar ratio of trehalose or sodium chloride, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0. The clarity of the 500:1 and 1000:1 molar ratio of sodium chloride to rhuMAb CD20 formulations was not measured after four weeks at 40°C due to the physical appearance of these formulations. The 500:1 ratio formulation was very opalescent while the 1000:1 ratio formulation had separated into two phases composed of a thin opaque gel layer covered with an opalescent fluid. The O.D. of the 1000:1 molar ratio of sodium chloride to rhuMAb CD20 formulation was 2.72 after two weeks storage at 40°C.

Figure 27 depicts the effect of excess molar ratios of trehalose on the stability of rhuMAb CD20 multidose formulations stored at 40°C as analyzed by SEC HPLC. The composition of the formulations is 40 mg/mL rhuMAb CD20, 20 mM acetate, 0-267 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0. The amount of trehalose in formulations 1-4 was as follows: 1 = 0 moles trehalose: 1 mole of rhuMAb CD20 (0 mM trehalose); 2 = 250 moles trehalose: 1 mole rhuMAb CD20 (67 mM trehalose); 3 = 500 moles trehalose: 1 mole rhuMAb CD20 (134 mM trehalose); and 4 = 1000 moles trehalose: 1 mole rhuMAb CD20 (267 mM trehalose). The percent monomer at each timepoint was normalized to the percent monomer at T=0.

Figure 28 shows the stability profile of the prototype liquid rhuMAb CD20 multidose formulation stored at 2-8°C for up to two years as measured by SEC HPLC. The formulation was composed of 40 mg/mL rhuMAb CD20, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0. The percent monomer at each timepoint was normalized to the percent monomer at T=0. The bioactivity of the formulation stored at 2-8°C for two years was 99.2% relative to the reference control as measured by the CDC assay.

Detailed Description of the Preferred Embodiments

I. Definitions

The term "pharmaceutical formulation" refers to preparations which are in such form as to permit the biological activity of the active ingredients to be unequivocally effective, and which contain no additional components which are toxic to the subjects to which the formulation would be administered.

"Pharmaceutically acceptable" excipients (vehicles, additives) are those which can reasonably be administered to a subject mammal to provide an effective dose of the active ingredient employed.

A "stable" formulation is one in which the protein therein essentially retains its physical stability and/or chemical stability and/or biological activity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in *Peptide and Protein Drug Delivery*, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, New York, Pubs. (1991) and Jones, A. *Adv. Drug Delivery Rev.* 10: 29-90 (1993), for example. Stability can be measured at a

selected temperature for a selected time period. Preferably, the formulation is stable at room temperature (~30°C) or at 40°C for at least 1 month and/or stable at about 2-8°C for at least 1 year and preferably for at least 2 years. Furthermore, the formulation is preferably stable following freezing (to, *e.g.*, -70°C) and thawing of the formulation.

A protein "retains its physical stability" in a pharmaceutical formulation if it shows no signs of aggregation, precipitation and/or denaturation upon visual examination of color and/or clarity, or as measured by UV light scattering or by size exclusion chromatography.

A protein "retains its chemical stability" in a pharmaceutical formulation, if the chemical stability at a given time is such that the protein is considered to still retain its biological activity as defined below. Chemical stability can be assessed by detecting and quantifying chemically altered forms of the protein. Chemical alteration may involve size modification (*e.g.* clipping) which can be evaluated using size exclusion chromatography, SDS-PAGE and/or matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI/TOF MS), for example. Other types of chemical alteration include charge alteration (*e.g.* occurring as a result of deamidation) which can be evaluated by ion-exchange chromatography, for example.

An antibody "retains its biological activity" in a pharmaceutical formulation, if the biological activity of the antibody at a given time is within about 10% (within the errors of the assay) of the biological activity exhibited at the time the pharmaceutical formulation was prepared as determined in an antigen binding assay, for example. Other "biological activity" assays for antibodies are elaborated hereinbelow.

By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

A "polyol" is a substance with multiple hydroxyl groups, and includes sugars (reducing and nonreducing sugars), sugar alcohols and sugar acids. Preferred polyols herein have a molecular weight which is less than about 600kD (*e.g.* in the range from about 120 to about 400 kD). A "reducing sugar" is one which contains a hemiacetal group that can reduce metal ions or react covalently with lysine and other amino groups in proteins and a "nonreducing sugar" is one which does not have these properties of a reducing sugar. Examples of reducing sugars are fructose, mannose, maltose, lactose, arabinose, xylose, ribose, rhamnose, galactose and glucose. Nonreducing sugars include sucrose, trehalose, sorbose, melezitose and raffinose. Mannitol, xylitol, erythritol, threitol, sorbitol and glycerol are examples of sugar alcohols. As to sugar acids, these include L-gluconate and metallic salts thereof. Where it desired that the formulation is freeze-thaw stable, the polyol is preferably one which does not crystallize at freezing temperatures (*e.g.* -20°C) such that it destabilizes the antibody in the formulation. Nonreducing sugars such as sucrose and trehalose are the preferred polyols herein, with trehalose being preferred over sucrose, because of the superior solution stability of trehalose.

As used herein, "buffer" refers to a buffered solution that resists changes in pH by the action of its acid-base conjugate components. The buffer of this invention has a pH in the range from about

4.5 to about 6.0; preferably from about 4.8 to about 5.5; and most preferably has a pH of about 5.0. Examples of buffers that will control the pH in this range include acetate (*e.g.* sodium acetate), succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers. Where a freeze-thaw stable formulation is desired, the buffer is preferably not phosphate.

In a pharmacological sense, in the context of the present invention, a "therapeutically effective amount" of an antibody refers to an amount effective in the prevention or treatment of a disorder for the treatment of which the antibody is effective. A "disorder" is any condition that would benefit from treatment with the antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

A "preservative" is a compound which can be included in the formulation to essentially reduce bacterial action therein, thus facilitating the production of a multi-use formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol. The most preferred preservative herein is benzyl alcohol.

As used herein, the term "inflammatory disorders" refers to pathological states resulting in inflammation, *e.g.* caused by influx of leukocytes and/or neutrophil chemotaxis. Inflammation may result from infection with pathogenic organisms and viruses and noninfectious means such as trauma or reperfusion following myocardial infarction or stroke, immune response to foreign antigen and autoimmune responses. Examples of inflammatory disorders include inflammatory skin diseases such as psoriasis and dermatitis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); ischemic reperfusion; adult respiratory distress syndrome; meningitis; encephalitis; uveitis; autoimmune diseases such as rheumatoid arthritis, Sjorgen's syndrome, vasculitis; diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome secondary to septicaemia or trauma; alcoholic hepatitis; bacterial pneumonia; antigen-antibody complex mediated diseases; hypovolemic shock; glomerulonephritis; multiple sclerosis; Type I diabetes melitis; acute and delayed hypersensitivity; graft vs. host disease; transplant rejection; reperfusion injury; endotoxic shock; disease states due to leukocyte dyscrasia and metastasis; asthma; pulmonary oxygen toxicity; inflammation of the lung, including pleurisy, alveolitis, vasculitis, pneumonia, chronic bronchitis, bronchiectasis, and cystic fibrosis; etc.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv

fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature* 352:624-628 (1991) and Marks *et al., J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*i.e.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined. The CDR and FR residues of the H52 antibody of the example below are identified in Eigenbrot *et al. Proteins: Structure, Function and Genetics* 18:49-62 (1994).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, FR residues of the human immunoglobulin are replaced by

corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); Riechmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

The antibody which is formulated is preferably essentially pure and desirably essentially homogeneous (*i.e.* free from contaminating proteins etc). "Essentially pure" antibody means a composition comprising at least about 90% by weight of the antibody, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" antibody means a composition comprising at least about 99% by weight of antibody, based on total weight of the composition.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

II. Modes for Carrying out the Invention

The invention herein relates to a stable aqueous formulation comprising an antibody. The antibody in the formulation is prepared using techniques available in the art for generating antibodies, exemplary methods of which are described in more detail in the following sections.

The antibody is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see US Patent 5,091,178) are also contemplated.

Where the antigen is a polypeptide, it may be a transmembrane molecule (*e.g.* receptor) or ligand such as a growth factor. Exemplary antigens include molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), *e.g.*, M-CSF, GM-CSF, and G-CSF; interleukins (ILs), *e.g.*, IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

Preferred molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and αv/β3 integrin including either α or β subunits thereof (*e.g.* anti-

CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; an interleukin such as IL8; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; *mpl* receptor; CTLA-4; protein C etc.

The preferred antibody herein is one which binds to human CD18 and preferably blocks (partially or completely) the ability of a cell (*e.g.* a neutrophil) expressing the CD18 subunit at its cell surface to bind to endothelium. Examples of anti-CD18 antibodies include MHM23 (Hildreth *et al., Eur. J. Immunol.* 13:202-208 (1983)); M18/2 (IgG_{2a}; Sanches-Madrid *et al., J. Exp. Med.*158:586 (1983)); H52 (American Type Culture Collection (ATCC) Deposit HB 10160); Mas191c and IOT18 (Vermot Desroches *et al., Scand. J. Immunol.* 33:277-286 (1991)); and NA-8 (WO 94/12214). The preferred antibody is one which binds to the CD18 epitope to which either MHM23 or H52 binds. In certain embodiments, the antibody may bind to a region in the extracellular domain of CD18 which associates with CD11b and the antibody may also dissociate α and β chains (*e.g.* the antibody may dissociate the CD11b and CD18 complex as is the case for the MHM23 antibody).

Techniques for producing antibodies which can be formulated as disclosed herein will be elaborated below.

A. Antibody Preparation

(i) Antigen preparation

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (*e.g.* the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (*e.g.* cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule. Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

(ii) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made

in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) Monoclonal antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro.* Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al., Nature*, 348:552-554 (1990). Clackson *et al., Nature*, 352:624-628 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al., Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al., Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, *et al., Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iv) Humanized and human antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al., Nature*, 321:522-525 (1986); Riechmann *et al., Nature*, 332:323-327 (1988); Verhoeyen *et al., Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library

of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al., J. Immunol.*, 151:2296 (1993); Chothia *et al., J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al., Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al., J. Immnol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993); and Duchosal *et al. Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom *et al., J. Mol. Biol.*, 227:381 (1991); Marks *et al., J. Mol. Biol.*, 222:581-597 (1991); Vaughan *et al. Nature Biotech* 14:309 (1996)).

(v) Antibody fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al., Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al., Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, Bio/Technology 10:163-167 (1992)). In another embodiment as described in the example below, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, $F(ab')_2$

fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(vi) Multispecific antibodies

Multispecific antibodies have binding specificities for at least two different epitopes, where the epitopes are usually from different antigens. While such molecules normally will only bind two different epitopes (*i.e.* bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Examples of BsAbs include those with one arm directed against a tumor cell antigen and the other arm directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-p185^{HER2}/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-p185^{HER2}, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell ahesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-α(IFN-α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA); BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (*e.g.* FcγRI, FcγRII or FcγRIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-FcγR/anti-HIV; BsAbs for tumor detection *in vitro* or *in vivo* such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-p185^{HER2}/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-HRP/anti-FITC, anti-CEA/anti-β-galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* F(ab')$_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al., Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker *et al., EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain

sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al.*, *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB)

disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby *et al., J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* 147: 60 (1991).

(vii) Effector function engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al., J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al. Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al. Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Antibody-salvage receptor binding epitope fusions.

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be

desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (*e.g.* by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, *e.g.*, by DNA or peptide synthesis).

The salvage receptor binding epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (*e.g.*, of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNSSMISNTP (SEQ ID NO:4), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:5), HQNLSDGK (SEQ ID NO:6), HQNISDGK (SEQ ID NO:7), or VISSHLGQ (SEQ ID NO:8), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s)(5' to 3'): HQNLSDGK (SEQ ID NO:6), HQNISDGK (SEQ ID NO:7), or VISSHLGQ (SEQ ID NO:8) and the sequence: PKNSSMISNTP (SEQ ID NO:4).

(ix) Other covalent modifications of the antibody

Covalent modifications of the antibody are included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues. Examples of covalent modifications are described in US Patent 5,534,615, specifically incorporated herein by reference. A preferred type of covalent modification of the antibody comprises linking the antibody to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

(x) Selecting biologically active antibodies

Antibodies produced as described above may be subjected to one or more "biological activity" assays to select an antibody with beneficial properties from a therapeutic perspective.

The antibody may be screened for its ability to bind the antigen against which it was raised. In the case of an anti-CD18 antibody, as shown in the example below, the antigen binding properties of the antibody can be evaluated in a "MAC-1 capture assay". Briefly, the assay involves first coating ELISA plates with an anti-CD18 antibody that binds to a site of MAC-1 different from the anti-CD18 antibody of interest to capture a recombinant preparation of soluble MAC-1, followed by a wash, addition of the sample to be tested, wash, addition of goat HRP-labeled anti-human $F(ab')_2$ antibody and colorimetric detection of OPD substrate. The total amount of antibody may

be measured by first coating an ELISA plate with a polyclonal anti-F(ab')$_2$ antibody, followed by addition of the sample, and then HRP-anti-F(ab')$_2$ and colorimetric detection of the HRP substrate, OPD. Specific activity is the ratio of the MAC-1 binding to total F(ab')$_2$ ELISA value.

In another embodiment, the affinity of the antibody may be determined by saturation binding; ELISA; and/or competition assays (*e.g.* RIA's), for example.

Also, the antibody may be subjected to other biological activity assays, *e.g.*, in order to evaluate its effectiveness as a therapeutic. Such assays are known in the art and depend on the target antigen and intended use for the antibody.

Where the antibody binds CD18, examples of biological activity assays include a slide adhesion assay, phagocytosis assay, neutrophil binding assay and degranulation assay.

The slide adhesion assay involves preincubating heparinized blood with various concentrations of anti-CD18 antibody, and then placing aliquots onto chambered glass slides. The slides are incubated at 37^{o}C to allow cells to adhere, nonadherent cells are gently washed off, the adherent cells are stained, and the average number of adherent cells per microscope field is determined for 30 fields.

For the phagocytosis assay, heparinized whole blood is obtained and the red blood cells are lysed. The cells are then incubated with opsonized BODIPY-labeled Staphylococcus aureus particles for 30 min at 37^{o}C. The cells are then analyzed by FACS, and the fluorescence intensity in the neutrophil gate is determined as an indication of the extent of phagocytosis.

For determining binding of anti-CD18 antibody to neutrophils, whole blood is incubated with various concentrations of the anti-CD18 antibody. The cells are then stained with a FITC-conjugated goat anti-human F(ab')$_2$ antibody, the red blood cells are lysed, and the white blood cells are analyzed by FACS. The fluorescence intensity in the neutrophil gate is proportional to the extent of anti-CD18 antibody binding.

For a degranulation assay, neutrophils are isolated from whole blood and preincubated with anti-CD18 antibody. The cells are then stimulated with opsonized zymosan particles and allowed to stand at room temperature. The cellular supernatants are then collected, and degranulation is assessed either by specific ELISA (for myeloperoxidase or lactoferrin) or by enzyme assay (for elastase).

For other antibodies, examples of biological activity assays include tumor cell growth inhibition assays (as described in WO 89/06692, for example); antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC) assays (US Patent 5,500,362); and agonistic activity or hematopoiesis assays (see WO 95/27062).

To screen for antibodies which bind to a particular epitope on the antigen of interest (*e.g.*, those which block binding of the humanized H52 antibody of the example to CD18), a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping, *e.g.* as described in Champe *et al., J. Biol. Chem.* 270:1388-1394 (1995), can be performed to determine whether the antibody binds an epitope of interest.

B. Vectors, Host Cells and Recombinant Methods

For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA

encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence (*e.g.* as described in US Patent 5,534,615, specifically incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K . thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g.*, *Neurospora*, *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger.*

Suitable host cells for the expression of glycosylated antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather,

Biol. Reprod. 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al., Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells, is removed, for example, by centrifugation or ultrafiltration. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al., EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and

shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

C. Preparation of the Formulation

After preparation of the antibody of interest as described above, the pharmaceutical formulation comprising it is prepared. The antibody to be formulated has not been subjected to prior lyophilization and the formulation of interest herein is an aqueous formulation. Preferably the antibody in the formulation is an antibody fragment, such as an $F(ab')_2$, in which case problems that may not occur for the full length antibody (such as clipping of the antibody to Fab) may need to be addressed. The therapeutically effective amount of antibody present in the formulation is determined by taking into account the desired dose volumes and mode(s) of administration, for example. From about 0.1 mg/mL to about 50 mg/mL, preferably from about 0.5 mg/mL to about 25 mg/mL and most preferably from about 2 mg/mL to about 10 mg/mL is an exemplary antibody concentration in the formulation.

An aqueous formulation is prepared comprising the antibody in a pH-buffered solution The buffer of this invention has a pH in the range from about 4.5 to about 6.0, preferably from about 4.8 to about 5.5, and most preferably has a pH of about 5.0. Examples of buffers that will control the pH within this range include acetate (*e.g.* sodium acetate), succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers. The buffer concentration can be from about 1 mM to about 50 mM, preferably from about 5 mM to about 30 mM, depending, for example, on the buffer and the desired isotonicity of the formulation. The preferred buffer is sodium acetate (about 10mM), pH 5.0.

An polyol, which acts as a tonicifier and may stabilize the antibody, is included in the formulation. In preferred embodiments, the formulation does not contain a tonicifying amount of a salt such as sodium chloride, as this may cause the antibody to precipitate and/or may result in oxidation at low pH. In preferred embodiments, the polyol is a nonreducing sugar, such as sucrose or trehalose. The polyol is added to the formulation in an amount which may vary with respect to the desired isotonicity of the formulation. Preferably the aqueous formulation is isotonic, in which case suitable concentrations of the polyol in the formulation are in the range from about 1% to about 15% w/v, preferably in the range from about 2% to about 10% w/v, for example. However, hypertonic or hypotonic formulations may also be suitable. The amount of polyol added may also alter with respect to the molecular weight of the polyol. For example, a lower amount of a monosaccharide (*e.g.* mannitol) may be added, compared to a disaccharide (such as trehalose).

A surfactant is also added to the antibody formulation. Exemplary surfactants include nonionic surfactants such as polysorbates (*e.g.* polysorbates 20, 80 etc) or poloxamers (*e.g.* poloxamer 188). The amount of surfactant added is such that it reduces aggregation of the formulated antibody and/or minimizes the formation of particulates in the formulation and/or reduces adsorption. For example,

the surfactant may be present in the formulation in an amount from about 0.001% to about 0.5%, preferably from about 0.005% to about 0.2% and most preferably from about 0.01% to about 0.1%.

In one embodiment, the formulation contains the above-identified agents (*i.e.* antibody, buffer, polyol and surfactant) and is essentially free of one or more preservatives, such as benzyl alcohol, phenol, m-cresol, chlorobutanol and benzethonium Cl. In another embodiment, a preservative may be included in the formulation, particularly where the formulation is a multidose formulation. The concentration of preservative may be in the range from about 0.1% to about .2%, most preferably from about 0.5% to about 1%. One or more other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980) may be included in the formulation provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include; additional buffering agents; co-solvents; antioxidants including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (*e.g.* Zn-protein complexes); biodegradable polymers such as polyesters; and/or salt-forming counterions such as sodium.

The formulation herein may also contain more than one protein as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect the other protein. For example, where the antibody is anti-CD18, it may be desirable to provide a further anti-adhesion antibody, such as an anti-ICAM-1 or anti-CD11a antibody along with the anti-CD18 antibody in a single formulation. Alternatively, the anti-CD18 antibody may be combined with another anti-inflammatory agent or a thrombolytic agent. Such proteins are suitably present in combination in amounts that are effective for the purpose intended.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to, or following, preparation of the formulation.

D. Administration of the Formulation

The formulation is administered to a mammal in need of treatment with the antibody, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. In preferred embodiments, the formulation is administered to the mammal by intravenous administration. For such purposes, the formulation may be injected using a syringe or via an IV line, for example.

The appropriate dosage ("therapeutically effective amount") of the antibody will depend, for example, on the condition to be treated, the severity and course of the condition, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, the type of antibody used, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments and may be administered to the patient at any time from diagnosis onwards. The antibody may be administered as the sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question.

As a general proposition, the therapeutically effective amount of the antibody administered will be in the range of about 0.1 to about 50 mg/kg of patient body weight whether by one or more administrations, with the typical range of antibody used being about 0.3 to about 20 mg/kg, more preferably about 0.3 to about 15 mg/kg, administered daily, for example. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques.

In the case of an anti-CD18 antibody, a therapeutically effective amount of the antibody may be administered to treat inflammatory disorders such as hemorrhagic shock, thermal injury (such as that resulting from burns), stroke (including ischemic and hemorrhagic stroke), and myocardial infarction. Where the antibody is an anti-IL8 antibody, the disorder may be an inflammatory disorder such as adult respiratory distress syndrome (ARDS), hypovolemic shock, ulcerative colitis, and rheumatoid arthritis.

E. Articles of Manufacture

In another embodiment of the invention, an article of manufacture is provided comprising a container which holds the aqueous pharmaceutical formulation of the present invention and optionally provides instructions for its use. Suitable containers include, for example, bottles, vials and syringes. The container may be formed from a variety of materials such as glass or plastic. An exemplary container is a 3-20cc single use glass vial. Alternatively, for a multidose formulation, the container may be 3-100cc glass vial. The container holds the formulation and the label on, or associated with, the container may indicate directions for use. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. All literature and patent citations are incorporated herein by reference.

EXAMPLE 1

This example describes an aqueous formulation comprising the antibody, recombinant humanized anti-CD18 antibody (rhuMAb CD18). RhuMAb CD18 having the amino acid sequence shown in Figure 1A (heavy chain; SEQ ID NO:1) and Figure 1B (light chain; SEQ ID NO:2) was created by humanization of the murine monoclonal antibody muMAb H52 (Hildreth *et al. J. Immunology* 134:3272-3280 (1985)).

The rhuMAb CD18 was produced recombinantly as described below. Plasmid pS1130 was constructed to direct production of a rhuMAb CD18 precursor molecule with a leucine zipper domain in *E. coli*. The precursor is cleaved during the purification process by the protease pepsin to yield rhuMAb CD18. rhuMAb CD18 is an $F(ab')_2$ molecule composed of two different peptides (light and heavy chains) linked by disulfide bonds. Fusion of a yeast GCN4 leucine zipper dimerization domain to the C-terminus of an Fab' substitutes for the Fc region and allows for efficient $F(ab')_2$ production in *E. coli*. The GCN4 leucine zipper domains interact to form stable dimeric structures (parallel coiled coils) that hold the hinge region cysteine residues of two heavy chains together so that the two native interchain disulfide bonds can form. This results in formation of $F(ab')_2$ complexes that are covalently linked by disulfide bonds. The leucine zipper domains are later removed from the rhuMAb CD18

precursor during the purification process using the protease pepsin, which cleaves uniformly between the two leucine residues of the hinge. This results in the formation of the rhuMAb CD18 $F(ab')_2$ molecule.

Plasmid pS1130 (Figure 20) is based on the well characterized plasmid pBR322 with a 2143 bp expression cassette (Figures 21A and 21B) inserted into the EcoRI restriction site. Plasmid pS1130 is resistant to both tetracycline and β-lactam antibiotics. The expression cassette contains a single copy of each gene linked in tandem. Transcription of each gene into a single dicistronic mRNA is directed by the *E. coli* phoA promoter (Chang *et al. Gene* 44:121-125 (1986)) and ends at the phage lamda t_0 terminator (Scholtissek and Grosse *Nucleic Acids Research* 15:3185 (1987)). Translation initiation signals for each chain are provided by *E. coli* STII (heat stable enterotoxin) (Picken *et al. Infection and Immunity* 42:269-275 (1983)) Shine-Dalgarno sequences. Translation of each chain begins with a 23 residue STII signal peptide that directs translocation of the peptides across the cytoplasmic membrane into the periplasmic space. The STII signal peptide is then removed by the *E. coli* leader peptidase. The light and heavy chains fold into their native conformations after secretion into the periplasm and associate into the rhuMAb CD18 precursor, a covalently linked $F(ab')_2$. The leucine zipper domain is cleaved from the precursor during the purification process (see below) to yield rhuMAb CD18. The cell line used in the production of rhuMAb CD18 is 49A5, derived from *E.* coli cell line W3110 (ATCC 27,325) as shown in Figure 22. The fermentation procedure takes place as shown in Figure 23. Production of rhuMAb CD18 precursor occurs when the medium becomes depleted in phosphate, typically 30-60 hours after inoculation.

Purification of rhuMAb CD18 precursor from the *E.* coli cell paste was as follows. Frozen cell pellets containing anti-CD18 precursor antibody, were dissolved in about 3 volumes of extraction buffer (120 mM MES, 5mM EDTA buffer, pH 6) heated to 30-40°C. This resulted in a suspension with a pH between about 5.4 and 6.5. This suspension was passed twice through a Gaulin homogenizer at 5500 to 6500 psi and kept below 20°C with a heat exchanger. 5% polyethyleineimine (PEI) (w/v), pH 6 was added to the homogenate to a final concentration of 0.2 % PEI. The mixture was incubated for about one hour at 2-8°C. About one volume of extraction buffer (120 mM MES, 5 mM EDTA, pH 6) was added before the solids were removed by centrifugation at 15,280g. The clear supernatant was conditioned to a conductivity of less than 3mohms by the addition of cold water. The conditioned supernatant was loaded onto a cation exchange column (ABX column; Mallinckrodt Baker, Inc., NJ, USA) equilibrated in 50 mM MES, pH 6.0. sodium citrate, pH 6.0. The cation exchange anti-CD18 precursor antibody pool was diluted with 50 mM MES, 36 mM sodium citrate, pH 4.0 to a concentration of approximately 2 g/L. The pool was then adjusted to pH 4 by addition of 2 M citric acid and flowed through a column containing immobilized pepsin (pepsin-CPG) previously equilibrated with 50mM MES, 36mM sodium citrate pH 4.0. Pepsin (Sigma, MO, USA) was chemically coupled to controlled pore glass (CPG) by Bioprocess Ltd., NJ, USA; the CPG was activated with NaIO4 followed by reduction of schiff base formation between CPG and pepsin using NaBH3CN). This procedure removed the zippers from the hinge region while leaving intact $F(ab')_2$. The effluent from the pepsin-CPG column was filtered directly in line through an anion exchange Sartobind Q filter (Sartorius, Goettingen, West Germany). The generated anti-CD18 $F(ab')_2$ antibody flows through the filter while pepsin and other negatively charge impurities bind strongly to the filter. The pool was diluted to give

a conductivity of approx. 7mohms by the addition of water and was then applied to a cation exchange column (SP Sepharose HP) equilibrated was 25mM MES, 60mM acetic acid, pH 4.0. The SP Sepharose column was washed with 25 mN MES, 75 MM sodium acetate pH 5.6 and eluted in a linear gradient of 75-110 mM sodium acetate in 25 mN MES pH 5.6. The pooled fraction from the SP sepharose column was diluted with the addition of 3.0M ammonium sulphate, 25 mM MES pH 6.0 at a ratio of 0.26 liters per liter of pool and was then passed through a hydrophobic interaction chromatography (HIC) column (phenyl sepharose FF-low substitution) previously equilibrated in 0.625 M ammonium sulphate, 25 nM MES pH 6.0. After loading, the column was washed with same buffer used in equilibration and the rhuMAb CD18 was eluted in 0.375M ammonium sulphate, 25 mN MES pH 6.0.

This resulted in purified rhuMAb CD18 for formulating as described below. Five aqueous formulations were evaluated for selection of a clinical formulation for use in, *e.g.*, hemorrhagic shock. The formulations were as shown in Table 1 below.

Table 1
Matrix of formulations prepared for this study.

Formulation	Buffer	pH	TWEEN 20™ (0.01 % v/v)	NaCl (140 mM)	Mannitol (4 % w/v)	Trehalose (8 % w/v)
F1	10 mM Acetate	5.0	+	+		
F2	10 mM Acetate	5.0	+			+
F3	10 mM Histidine	6.0	+	+		
F4	10 mM Histidine	6.0	+		+	
F5	10 mM Histidine	6.0	+			+

Two pH's were compared, pH 5 (sodium acetate) and pH 6 (histidine). At each pH, sodium chloride and trehalose were compared as tonicifiers. A histidine formulation with mannitol at pH 6 was also evaluated. The presence of a surfactant (*e.g.* 0.01% TWEEN 20™) was found to be required for stabilization against shaker-induced aggregation and for preventing adsorption to containers at protein concentrations below 1mg/mL. The stability assays indicated that the trehalose formulations were generally superior to the others, with pH 5 showing slightly greater chemical stability than the pH 6 formulation due to reduced clipping. Kinetic data after 2 weeks and 5 weeks storage at 5, 30, and 40°C were used for Arrhenius analysis and shelf-life prediction. Using cation exchange HPLC, the preferred formulation for rhuMAb CD18 was 10mM sodium acetate, 8% trehalose w/v, 0.01% TWEEN 20™, pH 5.0, with a predicted shelf life of 25-75 months (95% confidence intervals) at 5°C.

In designing antibody formulations, it may be useful to analyze the structural properties of the antibody to be formulated, but this is not necessary. RhuMAb CD18 consists of beta sheets of light (left

side of the molecule in Figure 16) and heavy chains (right side of the molecule in Figure 16) which are structurally stabilized each by two intramolecular disulfides and held together by two intermolecular disulfides (depicted as barrels at the bottom of Figure 16). The CDRs (complimentarity determining regions) in the variable segments are oriented on the top of the molecule. Located within and close to the CDRs are a number of methionines, only one of which (Met 65) is fully exposed. Though Met 65 is buried in anti-CD18, it is near a histidine residue which, without being limited to any one theory, might promote metal induced oxidation of proximal methionine (Li *et al. Biochem.* 34(17):5762-5772 (1995)). Susceptibility to oxidation in the CDRs was assessed herein. It was found to be desirable to use a sugar as the tonicifier, rather than a salt, so as to minimize oxidation at low pH.

In assessing the potential deamidation or isomerization sites in this molecule, *i.e.* Asx-Gly or Asx-Ser (Asx indicates Asp or Asn) that are in hydrophilic and flexible regions (Clarke *et al.* in *Stability of Protein Pharmaceuticals. Part A. Chemical and Physical Pathways of Protein Degradation*, T.J. Ahern and M.C. Manning, Editors. 1992, Plenum Press: New York. p. 2-29; and Kossiakoff, *Science*, 240:191-94 (1988)), six were found to be exposed and in fairly flexible regions (Figures 2A and 2B). Without being limited to any one theory, heavy chain motifs $MN^{84}S$, $KN^{55}G$, and light chain motifs $GN^{158}S$, $QD^{167}S$, $KD^{170}S$, are predicted to be most reactive; reactivity at Asn 84 is most likely to affect the binding activity of the antibody since it is near the CDR's. Asn 103 was found to be the primary route of degradation (via deamidation).

MATERIALS AND METHODS

The materials used in the following methods were as follows: glacial acetic acid 99.9% (MW 60.05); concentrated NaOH 18.94N (50%w/w; MW 40.0); concentrated HCl 37.8% (12.44N; MW 36.46); histidine (MW 155.16); NaCl (MW 58.44g); pharmaceutical grade trehalose dihydrate (MW 342.31); D-mannitol (MW 182.17); low peroxide TWEEN 20™; rhuMAb anti-CD18 (~1.3 mg/mL in MOPS, Na acetate, pH 6.9).

Buffer preparation and formulation set-up: Two liters of the following stock buffers were prepared for dialysis and formulation:

0.2 M Sodium Acetate, pH 5: 22.7 mL acetic acid, 17 mL 50% NaOH, made up to 2 L with Milli-Q water. Final pH 4.98. The solution was sterile filtered into 2 L Nalgene bottles and stored at 5°C.

0.2 M Histidine, pH 6: 62.06 g of Histidine, 20 mL concentrated HCl, made up to 2 L in a volumetric flask. Final pH 5.92. The solution was sterile filtered into 2 L Nalgene bottles and stored at 5°C.

2 M NaCl: 116.88 g NaCl, made up to 1 L with Milli-Q water. The solution was sterile filtered into 1 L Nalgene bottles and stored at 5°C.

18% Mannitol: 180 g mannitol, made up to 1 L with Milli-Q water. The solution was sterile filtered into 1 L Nalgene bottles and stored at 5°C.

20% Trehalose: 400 g trehalose, made up to 2 L with Milli-Q water. Final pH was 6.4. The solution was sterile filtered into 2 L Nalgene bottles and stored at 5°C.

10% (v/v) TWEEN 20™: 10 mL of concentrated TWEEN 20™ was carefully removed and added to a 100 mL volumetric flask, diluted with Milli-Q water, and stirred until dissolved. Stored at 2-8°C in the dark.

Formulation: Table 2 below shows the preparation of the buffers against which the starting protein bulk was dialyzed for this study. Approximately 40 mL of the bulk rhuMAb CD18 (~1.3mg/mL protein concentration) was dialyzed (2 L for 4 hr at 5°C and ~2 L overnight) to solutions prepared as shown in Table 2. The dialyzate was filtered with 0.2 µM Nalgene cellulose acetate filter units and stored under aseptic conditions for use as blank for UV and HPLC analysis.

Table 2: Summary of formulation preparation

Formulation		0.2 M buffer (mL)	18% Mannitol (mL)	20% Trehalose (mL)	1M NaCl (mL)	Approx. Water (mL)	pH	final pH	Made up to (mL)
F1	Acet/5	200	-	-	560	3000	5.07	5.07	4000
F2	Acet/5	200		1600		2000	5.17*	5.07	4000
F3	His/6	200	-	-	560	3000	6.03	6.03	4000
F4	His/6	200	889		-	2800	5.85*	6.01	4000
F5	His/6	200	-	1600		2000	5.97	5.97	4000

*Formulation F2 was adjusted with acetic acid buffer made by diluting 286 µL of glacial acetic acid and 200 mL of the 20% trehalose and mixed with Milli-Q water to a final 500 mL. This is 10 mM acetic acid with 8% trehalose. Approximately 250 mL was required to adjust the pH as required. Formulation F4 was adjusted with 0.5 mL of 50% NaOH.

Some of the remaining dialysis buffer was used to prepare formulation vehicles by adding TWEEN 20™, as shown in Table 3.

Table 3: Summary of preparation of formulation vehicles

Vehicle	Dialysis Buffer (mL)	10% TWEEN 20™ (µL)	Final Vol. (mL)
F1	41.4	41	41.4
F2	41.5	42	41.5
F3	42.4	42	42.4
F4	42.2	42	42.2
F5	41.4	41	41.4

Table 4 below shows the final appearance, pH, and protein concentration of the dialyzed protein solutions.

Table 4: Summary of analysis of the dialyzed protein solutions

Formulation	OD280 diluted 1:1	Conc.= OD/1.32*	pH of protein solution	Vehicle pH	color/ appearance	Temp °C
F1	0.8239	1.248	5.09	5.09	CAC**	24.7
F2	1.1192	1.696	5.09	5.09	CAC	24.7
F3	0.84605	1.282	6.06	6.06	CAC	24.7
F4	0.89763	1.36	6.10	6.10	CAC	24.7
F5	1.116	1.69	5.91	5.91	CAC	24.7

* Extinction coefficient was later changed to 1.45 mL/mg/cm.
**CAC = clear and colorless.

These protein solutions were then diluted, using reserved dialysis buffer, to a final ~1mg/mL protein concentration, and TWEEN 20™ was added to final 0.01% (v/v), as shown in Table 5.

Table 5: Summary of final formulation concentration adjustment and concentration determination

Form. no.	Dialysis buffer (mL)	10% TWEEN 20™ (µL)	Dialy. Protein (mL)	Starting Conc. (mg/mL)	Final Vol. (mL)	*Final OD280	*Final Conc. (mg/mL)	*OD 340-360
F1	8.16	41	33.2	1.248	41.4	1.3297	1.008	0.011
F2	17.0	42	24.5	1.696	41.6	1.3332	1.010	0.017
F3	9.26	42	33.1	1.282	42.4	1.3138	0.995	0.009
F4	11.2	42	31	1.36	42.2	1.3065	0.990	0.010
F5	16.9	41	24.5	1.69	41.4	1.3118	0.994	0.016

* Measured after filtration and filling.

The formulations were sterile filtered using 150 mL Nalgene filter units with cellulose acetate membranes in a sterile hood. The sterile formulated rhuMAb CD18 solutions were filled at 0.6 mL per 3 cc vial, labeled and stored at designated temperatures. Vehicle solutions were filtered and filled identically. Tables 6A (antibody formulations) and 6B (vehicle controls) below show the stability program for these formulations. Data presented here are up to 5 weeks storage.

Table 6A - CD18 antibody formulations

time	40°C samples	30°C samples	5°C samples	-20°C samples	-70°C samples
t0			2	2	2
2 wk	2	2	2		
5 wk	2	2	2	2	2
8 wk	2	2	2		
12 wk	2	2	2	2	2
6 mo		2	2	2	2
9 mo - 1 yr		2	2	2	2
	8+2 ext=10	12	14	8	10+4ext=14

Table 6B - Vehicles

time	40°C vehicles	30°C vehicles	5°C vehicles	-20°C vehicles	-70°C vehicles
t0			1	1	1
2 wk	1	1	1		
5 wk	1	1	1	1	2
8 wk	1	1	1		
12 wk	1	1	1	1	2
6 mo		1	1	1	2
1 yr		1	1	1	2
	4	6	6+2=8	4+2=8	8+2ext

At the 2 week and 5 week timepoints, 2 vials from each formulation and storage temperature were frozen at -70°C. At the 5 week timepoint the frozen samples were thawed and batch analyzed together with control samples frozen at -70°C immediately after filling (t=0 weeks).

UV Spectroscopy: Samples were measured without dilution in a Hellma 2 mm wide, 1 cm path-length cell with raised bottom and black sides. Approximately 300µL of sample was used for the measurements. The instrument was blanked with Milli-Q water. Samples were scanned from 200 to 400 nm with a 2 nm bandwidth and 1 second integration using a HP-8452A spectrophotometer.

Protein concentration was determined from the absorbance at 278 nm correcting for the absorbance at 320 nm using an extinction coefficient of 1.45 mL/mg/cm. The average value of absorbances in the region of A_{340} to A_{360} was followed as an indicator of scattering material.

pH: An Orion 720A pH meter was used with a MicroElectrodes, Inc. MI-410 micro pH electrode. The electrode was calibrated with pH 4 and 7 standard buffers, re-checked every 10-15 samples, and recalibrated as required. The instrument was calibrated and samples were measured at room temperature (~23°C).

RP-HPLC: RP-HPLC was carried out using a 7.5 x 75 mm TSK Phenyl-5PW column with 0.1% TFA as the A buffer, and 0.08% TFA in acetonitrile as the B buffer. An inline 0.5µM filter was used before the column. The column was equilibrated at 10% B buffer and run at 55°C. Injections of 20µL were made and eluted according to Table 7 below:

Table 7

Time (Minutes)	%B	Flow (mL/minute)
0	10	0.8
30	25	0.8
35	70	0.8
35.01	70	2
36	70	2
37	10	2
41	10	2

Data collected at 214 nm at 1 point per second were used for analysis.

SEC Assay: The mobile phase used was 200 mM NaCl, 50 mM sodium phosphate at pH 6.0. The runs were 40 minutes long at a flowrate of 0.5 mL/minute with 40µL injections done at ambient temperature. A TSK G3000SWXL 30 cm x 7.8 mm column with inline filter and a TSK guard column before the main column was used. Detection was at 214 nm.

Ion-Exchange HPLC Assay (IEX): IEX was run using 50µL injections. The mobile phases were 33 mM each of MES/ HEPES/PIPES, adjusted to pH 6.0 for A and pH 8.0 for B; C= Milli-Q water. The gradient shown in Table 8 below was used:

Table 8

Time (min)	% B	% C	Flow (mL/min)
0	0	50	1
3	0	50	1
15	20	50	1
50	50	50	1
50.1	100	0	2
51	100	0	2
51.1	0	50	2
60	0	50	2

The method used a 50 x 4.6 mm BakerBond carboxy-sulphon (CSx) cation exchange column with an inline 0.5µM filter before the column, run at 40°C. Analysis was done with data obtained at 280 nm, due to the high buffer background.

Hydrophobic Interaction Chromatography (HIC): HIC was done on a 4.6 x 50mm Baker Bond Butyl NPR column with inline 0.5 µM filter before the column. The running buffers were 2M ammonium sulfate in 20 mM Tris-HCl, pH 7 as the A buffer, and 20 mM Tris, pH 7 as the B buffer. The column was equilibrated at 50°C with 10% buffer B at 1 mL/minute and 10 µL injections were made. The gradient shown in Table 9 below was used for elution:

Table 9

Time	%B
0	10
1	10
35	100
37	100
37.1	10
42	10

Data were collected at 214 nm at 1 point per second.

SDS-PAGE: Pre-made 10 and 14% glycine SDS-PAGE gels (Novex, San Diego, CA) were used for analysis of the reduced and non-reduced samples, respectively. 10 µL of the 1 mg/mL sample diluted with 10 µL of 2X Tris-Glycine sample buffer (Novex) was heated 2 minutes at 95°C, as were high MW range and low MW range (Bio-Rad) markers diluted 1:20 in same sample buffer. Kaleidoscope MW marker was heated at 95°C, as well, with no dilution. When the samples cooled, 10µL was loaded on the gels. The load of the MW marker solutions was 5µL. The gel apparatus (Novex) was filled with 1X gel loading buffer (Media Services) and the gels were run at constant voltage of 125 mV and variable current for 2 hours. The gels were stained with the Novex Coomassie blue stain, overnight. The next day, the gels were washed with water for 2 hours and then were soaked in Gel Drying Solution (1X) (Novex) for ½ hour. The gels were then air-dried with the Novex cellophane system.

Gel Isoelectric Focusing Electrophoresis: IEF was done using Pharmacia 3.5 to 9.5 PAG plate gels. Pharmacia phosphoric acid and sodium hydroxide running buffers were used. Samples were prepared by mixing 10 µL of sample with 30 µL of 15 % glycerol. Pharmacia broad range and high

range IEF standards, as well as Serva Protein Test Mix 9 were run as pI markers. Pharmacia standards were prepared by mixing the standard (reconstituted with 0.5 mL of Milli-Q water) 1:1 with 15 % glycerol. The Serva protein mix was reconstituted according to instructions and diluted 10-fold with 15 % glycerol. On one gel, anti-HER2 MAb bulk (pI = 8.8 - 9.0 calculated) was also run as a control. The HER2 sample (5 mg/mL) was diluted 40X with 15% glycerol. The gels were loaded with 20µL of each sample adsorbed onto paper patches placed one third of the gel width from the positive electrode (the acid side). Gels were run at 10°C with constant voltage set to 400 V for 30 minutes, after which the sample loading papers were removed and the gels were run for 1.5 more hours at 1500 V. Gels were fixed in TCA with sulfosalicylic acid for 30 minutes, and washed with water for 10 minutes (for the second gel) or IEF destain (for the third gel), and stained without washing out the TCA, as instructed in the Novex instructions (for the first gel). The first two gels were stained with Novex colliodal Coomassie according to the Novex instructions. The last gel was stained for 8 minutes with standard IEF Coomassie stain which was preheated to ~60°C, and then destained with IEF destain which had also been preheated to ~60°C overnight. The gels were air-dried with the method described above for the SDS-PAGE gels.

Freeze/Thaw Stability: To demonstrate that rhuMAb CD18 would not degrade by freeze/thaw cycling in the formulations being tested, four vials of each formulation were subjected to three cycles of freezing and thawing. Two of the vials of each formulation were cycled between -70°C and 2-8°C and two were cycled between -20°C and 2-8°C. Two vials of each formulation were held at 2-8 °C as controls. During each cycle the vials were allowed to remain in the freezer for at least 2 hours, until there was no visible liquid remaining in the vial. They were then transferred to 2-8°C for at least 2 hours, until no solid remained. One vial of the acetate/trehalose, pH 5 formulation (F2) did not freeze at -20°C on the first cycle, but froze immediately when placed on the shelf in a -70°C freezer.

Shaker Studies: One vial of each formulation, and a vial of identically configured rhuMAb CD18 bulk, was placed horizontally into a rack on a Glas-Col 99A S60012 shaker. The shaker arm was adjusted to a radius of ~30 cm and the speed was set to 70 rpm. The samples were shaken for 24 hours at room temperature in the axial direction. A second vial of each formulation, and the bulk, was maintained at room temperature near the shaker, as a control. The samples were then analyzed by IEX, MAC-1 capture assay, and UV spectroscopy.

MAC-1 Capture (Receptor ELISA) and Total F(ab')$_2$ ELISA: rhuMAb CD18 is directed to the CD18 chain of MAC-1. The antigen binding property of rhuMAb CD18 can hence be evaluated by "MAC-1 capture assay". The assay involves coating of a 96 well mictrotitre plate first with a murine anti-CD18 antibody that binds to a site of MAC-1 different from rhuMAb CD18 (MHM23 MAb; Hildreth *et al., Eur. J. Immunol.* 13:202-208 (1983)) to capture a recombinant preparation of soluble MAC-1, followed by a wash with phosphate buffered saline (PBS), addition of the unknown sample, wash with PBS, addition of goat HRP-labeled anti-human F(ab')$_2$ antibody and colorimetric detection of OPD substrate. The total amount of antibody is measured by first coating an ELISA plate with a polyclonal anti-F(ab')$_2$ antibody, followed by addition of the sample, and then HRP-anti F(ab')$_2$ and colorimetric detection of the HRP substrate OPD. Assay diluents in both cases were 0.5% bovine serum albumin (BSA), 0.05%

polysorbate 20, 1mM $CaCl_2$, 1mM $MgCl_2$. Specific activity was measured as the ratio of the MAC-1 binding to total $F(ab')_2$ ELISA value.

The samples were serially pre-diluted with assay diluent obtained to final dilutions of 20,000, 40,000, and 80,000 times in microtiter plates and Micronics tubes using multi-channel pipettors. The dilution scheme used was as shown in Table 10 below. Samples of diluted rhuMAb CD18 bulk were included on each assay plate as controls. These bulk controls were diluted one extra 2X dilution to final 40,000, 80,000, and 160,000 times. The same dilutions were used for both assays. Samples were diluted the same day as they were thawed for the 5 week analysis and then frozen in the Micronics tubes prior to submission for assay the following day. The freeze/thaw stability of rhuMAb CD18 diluted in assay diluent in these assays was verified.

Table 10

Step	Dilution (times)	Sample (µL)	Diluent (µL)
1, in 96 well plate*	10	10	90
2, in 96 well plate*	10	10	90
3, in 96 well plate*	10	10	90
4, in Micronics tubes	20	50	950
5, in Micronics tubes	2	500	500
6, in Micronics tubes	2	500	500

*Later studies were conducted by making all dilutions in Micronics tubes and vortexing them to mix; this improved reproducibility.

Kinetic Parameters, Arrhenius Calculations, and Statistics: The loss in the % main peak versus time was fitted to first order kinetics. The observed rate constants (κ) at various temperatures were then fitted to the Arrhenius equation as shown below:

$$\ln \kappa = -\Delta E/RT + C$$

where E is the activation energy (in cal/mol), R is the Universal gas constant (1.987 cal/mol/K), T is the absolute temperature (in Kelvins) and C is a constant. From the slope of a plot of ln κ vs. 1/T, ΔE was calculated. The standard error (SE) of ln κ was calculated using the formula $[(1/\kappa)^2 + (SE)^2]^{0.5}$. The reason for a large SE value for ln κ at lower temperatures is the small value of the κ which is in the denominator of the formula. A value of κ extrapolated from the Arrhenius fit (± 95% confidence interval) was used to predict the shelf life at 5°C according to:

$$t_{90}\ (wk) = \ln 0.9/\kappa$$

where κ was expressed in $weeks^{-1}$. Confidence intervals were determined using the program SIGMAPLOT™.

RESULTS AND DISCUSSION

Freeze/Thaw stability: Table 11 below shows a summary of the data from analysis of the samples in the freeze/thaw study. No significant differences were seen between the different temperature groups. The observation that one vial of F2 did not freeze at -20°C on the first cycle raised a concern that freezing of bulk in a jacketed stainless steel storage tank might be problematic. The coldest parts of the solution in these tanks reach only about -20°C, and therefore might not freeze completely. This was tested using formulation vehicle. The tank was observed to freeze solid within 6 hours of cooling, so no problem for bulk storage is predicted.

Table 11. Effect of three cycles of freezing and thawing to 5°C on the Stability of rhuMAb CD18

Formulation	RP-HPLC Total Area	RP-HPLC % Main	SEC Total Area	SEC % Main	UV Conc. (mg/mL)	UV Average 340 - 360 nm	MAC-1 Capture Titer (µg/mL)	Specific Activity (MAC-1/UV)
F1 5°C	10276±25	88.0±0.1	6401±151	83.8±0.4	0.949±0.003	0.011±0.001	1000±32	1.05±0.03
-20°C	10341±106	88.2±0.1	6556±73	83.2±0.1	0.952±0.002	0.012±0.001	948±28	1.00±0.03
-70°C	10328±13	87.5±0.01	6353±63	83.0±0.9	0.948±0.005	0.013±0.004	972±15	1.03±0.02
F2 5°C	10537±4	88.5±0.2	6508±18	81.7±0.3	0.941±0.006	0.017±0.005	1069±57	1.14±0.06
-20°C	10644±107	87.3±0.5	6290±54	83.1±0.2	0.941±0.002	0.012±0.001	1132±27	1.20±0.03
-70°C	10503±74	88.5±0.2	6435±120	82.5±0.8	0.938±0.000	0.009±0.002	1064±41	1.13±0.04
F3 5°C	10147±50	87.9±0.2	6311±65	84.8±0.3	0.926±0.002	0.017±0.001	1017±77	1.10±0.08
-20°C	10050±43	88.4±0.00	6404±39	85.6±0.1	0.922±0.007	0.012±0.001	997±28	1.08±0.03
-70°C	9953±85	88.0±0.2	6356±52	83.9±0.2	0.926±0.002	0.017±0.005	1021±26	1.10±0.03
F4 5°C	10397±26	88.2±0.1	6304±148	84.5±1.1	0.921±0.000	0.010±0.003	905±18	0.98±0.02
-20°C	10126±180	88.6±0.03	6467±13	83.5±0.2	0.922±0.004	0.014±0.001	1004±20	1.09±0.03
-70°C	10322±51	88.1±0.1	6327±3	84.7±1.1	0.924±0.004	0.009±0.007	982±21	1.06±0.03
F5 5°C	10438±81	88.0±0.1	6418±64	85.9±0.3	0.930±0.001	0.016±0.004	1058±39	1.14±0.04
-20°C	10443±10	88.3±0.1	6310±11	86.5±0.4	0.923±0.004	0.016±0.003	1010±49	1.09±0.05
-70°C	10350±135	88.3±0.00	6340±58	85.5±0.1	0.923±0.003	0.017±0.006	1003±22	1.09±0.02

Mean ± SE of two vials are shown. No significant loss in monomer content or receptor binding was noticed.

UV Spectroscopy: Spectroscopic analysis of the samples was done to measure protein concentration as well as to measure light scattering as indicated by an increase in average absorbances in the range of 340 to 360 nm, or by a decrease in the ratio of the absorption maxima at 278 nm and the minima at 252 nm. Figures 3A and 3B show a summary of the UV data. The 40°C samples of formulation F3 were very cloudy, containing white flocculent particles. These samples were microfuged for 5 minutes at 14000 RPM and re-measured after which the scatter was still relatively high. All other samples appeared clear and colorless ($A_{340-360} < 0.02$, $A_{278}/A_{252} > 2.4$). The change in A278/A250 was a more sensitive indicator of scattering than the average absorbances from 340 to 360nm, since the latter showed no storage temperature related trend (Figure 3A), but the former showed a definite increase for the 40°C samples (Figure 3B). The pH 5 formulations showed a smaller change in A278/A252 ratio at 40°C than the pH 6 formulations in every case. The protein concentrations for the pH 5 formulations were only 2-3 % greater than the pH 6 formulations (Figure 4).

pH: All formulation vehicles except F4 showed a slight increase (~0.1 units at 40°C) in pH, compared with -70°C controls (Table 12 below). Both pH 5 formulations showed a greater pH increase in the active samples than in the vehicles. None of the pH 6 formulations showed this difference between active and vehicle samples. In fact, the pH increase seen in the F3 and F5 vehicles was not seen in the active samples. The pH of F4 decreased slightly at higher storage temperature. The pH increase in the pH 5 samples could be explained by a loss in acetic acid concentration due to diffusion through the stopper, and is not expected to be significant at 5°C.

Table 12: Formulation pH after 5 weeks storage.

Sample	pH of Vehicle	pH of Active (both vials shown)
F1 at -70°C	4.892	5.009/5.026
F1 at -20°C	4.894	5.028/5.035
F1 at 5°C	4.947	5.077/5.082
F1 at 30°C	5.040	5.154/5.164
F1 at 40°C	5.045	5.171/5.171
F2 at -70°C	5.037	5.179/5.089
F2 at -20°C	5.041	5.096/5.101
F2 at 5°C	5.098	5.151/5.153
F2 at 30°C	5.134	5.192/5.213
F2 at 40°C	5.136	5.242/5.215
F3 at -70°C	6.123	6.164/6.154
F3 at -20°C	6.162	6.155/6.138
F3 at 5°C	6.160	6.130/6.086
F3 at 30°C	6.204	6.138/6.090
F3 at 40°C	6.214	6.098/6.075
F4 at -70°C	5.964	6.014/6.016
F4 at -20°C	6.017	6.050/6.019
F4 at 5°C	6.021	6.004/5.988
F4 at 30°C	5.928	5.914/5.897
F4 at 40°C	5.880	5.849/5.854
F5 at -70°C	5.653	5.751/5.754
F5 at -20°C	5.729	5.761/5.766
F5 at 5°C	5.727	5.768/5.777
F5 at 30°C	5.797	5.843/5.833
F5 at 40°C	5.812	5.822/5.831

The pH 5 formulations showed a small increase in pH at higher storage temperatures for the vehicles and active samples. Formulations F3 and F5 showed increasing pH in the vehicles with higher storage temperature, but the active samples did not show this increase. Formulation showed a slight reduction in pH.

SEC: Figures 5A and 5B show the chromatograms for formulations F2 (Figure 5A) and F5 (Figure 5B). The main peak of rhuMAb CD18 eluted at 18.6 minutes, corresponding to an apparent MW of ~70,000 D. There were two major impurity peaks that eluted after the main peak, and the one at 20.9 minutes increased considerably with increasing time and storage temperature. The increase in the peak at 20.9 minutes was much more pronounced in F5 (Figure 5B) compared to F2 (Figure 5A), without being limited to any one theory, most likely due to the higher pH of F5. Figures 6A and 6B show graphs of the total peak area and the % main peak for all of the formulations after 0, 2 and 5 weeks of storage at 5, 30 and 40°C. The total peak areas for formulations F3 and F4 were significantly lower than the other three formulations.

Since the lower pH formulations showed the greatest stability, rate data from the % main peak of the SEC chromatogram were calculated and fitted to an Arrhenius plot. Data for F2 is shown in Figure 7. This plot contained only two useful points since the rate constant at 5°C had a large standard error. The activation energy obtained from these data (19 ± 6 kcal/mol) was very similar to the one calculated from the IEX data (see Figure 15) even without the 5°C datum. The 95% confidence intervals to the curve fit predict a shelf-life from 6 to 60 years at 5°C.

HIC:This assay was performed at a column temperature of 50°C rather than 55°C to improve protein recovery (from ~30% to nearly 90%), unfortunately at the expense of loss in resolution. HIC analysis of the stability samples showed an increase in a poorly resolved peak on the leading shoulder of the main peak, as well as an increase in a 17.5 min peak with increasing storage time and temperature for all formulations (Figure 8). The shoulder was in a position that would be consistent with an oxidized form of the protein, though in preliminary assessment no peaks corresponding to an oxidized species appeared by peptide mapping. F2 showed the smallest increase in this leading peak (Figure 9). A trend towards reduced recovery (total area) was also seen with increasing storage temperatures, with all formulations showing the same total area at 40°C (Figure 10). Formulations F2 and F5 showed the least change in total area at 5 and 30°C relative to the starting -70°C sample. This method was not stability indicating because the resolution between the degradation peak at 17.5′ and the main peak is too low for accurate quantitation, and the recovery from the method is poor.

RP-HPLC: The only change observed by RP-HPLC was an increase in a peak eluting at about 25 minutes, with a corresponding reduction in the main peak area, as the storage temperature increased (Figures 11 and 12). The magnitude of this change was similar to that of the lower MW species seen in SEC. Based on the relative position of this peak and its area, it may correspond to the low MW species. Formulation F2 showed the least change in the % main peak area with increasing temperature. Samples reduced with DTT were also run on RP-HPLC to increase the chance that an oxidized form might be resolved from one of the separated smaller subunits of the antibody. No difference was seen between the stored samples and the -70°C control samples with this method.

The Arrhenius plot of the RP-HPLC kinetic data for formulation F2 gave a similar activation energy (21 ± 1 kcal/mole) to the SEC and IEX results (19 - 20 kcal/mole) (Figure 13). The shelf-life indicated by the 95% confidence intervals to the curve fit is 4 to 150 years at 5°C.

IEX: By this assay, earlier eluting peaks with retention times of 18.4 and 26.6 minutes increased with increasing storage temperature. As shown in Figures 14A and B which represent formulations F2 and F5, respectively, there was a more pronounced increase of these peaks in F5, compared to F2. Also the peak at 19.5 minutes became broader at higher temperatures. Based upon chromatographic changes seen upon exposure of samples to pH 11 (see below), this method should readily detect changes such as deamidation and/or disulfide scrambling, both of which can be induced at alkaline pH.

Table 13 below shows the first order rate constants for the 5 formulations at 5, 30, and 40°C.

Table 13: First order rate constants (κ ± SE; n=2) for all formulations based on % main of IEX

Formulation	5°C	30°C	40°C
F1	0.00064±0.0021	0.0083±0.0014	0.0299 ±0.0023
F2	0.0065±0.0023	0.0091±0.0025	0.0245±0.0040
F3	-0.0004±0.0008	0.0143±0.0010	0.0432±0.0019
F4	-0.00066±0.00073	0.0137±0.00033	0.0413±0.00022
F5	-0.00010±0.00082	0.0129±0.00095	0.0439±0.0017

Formulations F1 and F2 are clearly better than the other formulations, implying that the major degradation process is base catalyzed. An Arrhenius plot based on the % main peak area predicted an activation energy of 19.8 ± 9.9 kcal/mole for F2 and a t_{90} of 50 ± 25 months (95% confidence intervals) at 5°C (Figure 15).

Receptor binding activity: Table 14 below shows the specific activity for each temperature and formulation after 5 weeks storage. The specific activity was calculated by dividing the MAC-1 capture assay concentration by the total $F(ab)_2$ ELISA concentration. The standard error shown is for n=2 and accounts for the total propagated error from the two different assays for 2 vials at 3 dilutions each. There was a clear trend for all formulations towards a lower specific activity at higher storage temperatures (~10-15 % loss at 40°C); all formulations showed about the same extent of loss at 40°C relative to -70°C. The p value from one way ANOVA analysis of the 40°C data for the different formulations is 0.98, so the 40°C means would not be considered significantly different from each other, within 95% confidence. An unpaired t-test of the 40°C data versus the 30°C data shows that the difference is significant (p=0.044) and the lower temperatures are even more significantly different by this method (p < 0.02).

Table 14

Storage	F1		F2		F3		F4		F5	
Temp (°C)	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
-70	0.976	0.098	0.906	0.140	1.008	0.112	0.952	0.116	0.918	0.433
-20	0.978	0.123	0.903	0.157	0.946	0.152	0.871	0.072	0.865	0.209
5	0.982	0.157	0.898	0.177	1.023	0.166	0.919	0.203	0.886	0.350
30	0.923	0.067	0.878	0.189	0.949	0.076	0.868	0.109	0.885	0.340
40	0.866	0.113	0.817	0.227	0.904	0.115	0.817	0.093	0.772	0.198

IEF: The effect of storage for 5 weeks at different temperatures was assayed by isoelectric focusing electrophoresis (IEF) for formulations F2 and F5. The major band appeared to have a pI of ~8.8. Three main components at pI's ~8.75 (second most intense band), ~8.6, and ~8.4 were also seen, which increase slightly with storage, particularly at pH 6. The 30 and 40°C samples showed conversion to the more acidic bands. For the 40°C samples, the bands at pI 8.8 and 8.75 had approximately equal intensity and there was no discernible difference between the formulations in this regard. There were at least two more acidic bands (pI's ~8.6 and ~8.4), which had at least 4-fold lower intensity than the two most basic bands. These bands did not seem to change in intensity with storage.

SDS-PAGE: The effect of storage temperature on the stability of rhuMAb CD18 formulations F2 and F5 (5 weeks) was assayed by SDS-PAGE (under reduced and nonreduced conditions). The rhuMAb CD18 appeared at a MW of ~120 kD on the non-reduced gel. In the starting bulk there were several minor bands at higher molecular weight than the main band which may represent rhuMAb CD18 with various portions of the leucine-zipper segment still attached. Several percent of this impurity is known to be present in this bulk preparation. There were several bands of lower MW than the main band, but only one of these bands seemed to change in any of the formulations, or with temperature. The band at around 45 kD increased with temperature and seemed slightly more intense in pH 6 than in pH 5 formulations. This observation was consistent with SEC data. The band just above the ~45 kD band on the non-reduced gels corresponded in apparent molecular weight with the band seen on the reduced gels and may represent a non-reducing contaminant protein. All species converted to two bands, corresponding to light and heavy chain, upon reduction with dithiothreitol, suggesting the absence of any proteolytic cleavage. MALDI-TOF MS (Yates, J. *Methods Enzymol.*, 271:351-377 (1996)) confirmed formation of fragments that were the size of Fab'.

Formulation pH and protein concentration: The effect of pH on the conformation of rhuMAb CD18 was investigated using fluorescence spectroscopy (Figure 17). The protein appeared to lose tertiary structure below pH 3, but is unchanged in the range of pH 3 to 8.

Based on the above observation, the effect of pH and protein concentration on the stability of rhuMAb CD18 was then investigated using a central composite design protocol. Formulations containing 10 mM Na citrate, 8% trehalose, 0.05% TWEEN 20™ containing one of the following conditions were prepared: 0.5 mg/mL (pH 4.5), 5 mg/mL (pH 3, 4.5, 6), 10 mg/mL (pH 4, 5), 25 mg/mL (pH 4.5). Samples were placed at 40°C and -70°C, and analyzed after 2 month by IEX (Figure 18A) and SEC (Figure 18B). Again, pH 5 was found to be the most stable condition for the protein, irrespective of protein concentration.

Long Term Stability of rhuMAb CD18 Formulation: To address the long term stability of the F2 formulation, duplicate samples in 10 mM Na acetate, 8% trehalose, 0.01% TWEEN 20™, pH 5 were prepared in 3 cc glass vials and placed on stability at the indicated temperatures. Analysis by SEC (Figure 19A) and MAC-1 binding (Figure 19C) indicates no change in the size distribution, aggregation state, or bioactivity of the protein for up to 43 weeks at 5°C; stability up to ~ 1mo at 30°C is indicated. IEX shows formation of acidic peaks (~1% after 43 weeks) that were identified to be deamidation by peptide mapping/MS (Figure 19B).

CONCLUSIONS

Preliminary data suggested that the primary reaction in the purified rhuMAb CD18 was cleavage to species approximately half the MW of the starting material, and that this reaction, as well as generation of acidic peaks on IEX and earlier eluting peaks on HIC and RP-HPLC, were all minimized at pH 5 (compared to pH 6), and in trehalose (compared to salt or mannitol). The smaller MW species may have been formed either by proteolytic cleavage and/or by disulfide scrambling, both of which may be enhanced at higher pH's. Reduction of the control and degraded samples led to only the light and heavy chains, consistent with either the cleavage to Fab, or mixed disulfide formation between heavy and light chains.

RhuMAb CD18 appeared to be least stable in salt formulations; at pH 5 a larger aggregate (equivalent to a trimer) appeared and at pH 6 precipitation was noted at 40°C. Without being bound to any one theory, one possible explanation is that NaCl does not provide hydrogen bonding, as sugars and mannitol do, which could potentially prevent self-association of this antibody at higher temperatures.

Based on the above preliminary data from the primary assays (IEX, SEC, and UV), the preferred aqueous formulation for rhuMAb CD18 is 10 mM sodium acetate, 8% trehalose w/v, 0.01% TWEEN 20™, pH 5.0. The shelf-life predicted from an Arrhenius fit to the first order rate constants (IEX data) is 1.7 to 4 years at 5°C (95% confidence intervals).This formulation can be prepared by mixing 0.573 mL glacial acetic acid, 0.403 mL concentration NaOH, 80 g trehalose, 1 mL of a 10% TWEEN 20™ solution and making up to 1 L with MilliQ water (pH 5.0±0.1 at the 2 L scale). Conductivity of this formulation was found to be 502±10% microSiemens/cm using a Radiometer-Copenhagen CDM-83 with a CDC 314 probe, and density was 1.017 g/mL.

EXAMPLE 2

This example describes the production of a stable aqueous multidose formulation comprising a recombinant humanized anti-CD20 antibody, rhuMAb CD20. Acetate (pH 5) formulations stored at 40°C for one month demonstrated greater stability than those samples formulated in histidine (pH 5 or 6). The histidine formulations after accelerated temperature storage became very opalescent and yellow in color. A buffering capacity of 10-30 mM acetate was sufficient to maintain the pH at 5.0. The effective amount of tonicity modifier needed to stabilize the antibody against freeze or thermal induced aggregation was compared using sodium chloride (NaCl) or trehalose. Trehalose was found to protect the formulation from freeze induced aggregation, particularly at levels $\geq$ 134 mM (500:1 molar ratio). The trehalose formulations (67-270 mM) were much more effective than NaCl in stabilizing formulations placed at 40°C as evidenced by the clarity of the solution. These results led to the development of a stable prototype liquid multidose formulation comprising 40 mg/mL rhuMAb CD20, 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% polysorbate 20 at pH 5.0 that has a minimum shelf life of two years storage at 2-8°C.

MATERIALS AND METHODS

RhuMAb CD20 : The bulk material used in all studies was composed of 27 mg/mL rhuMAb CD20 in 50 mM Tris and 100 mM sodium chloride at pH 7.5 or 2.7 mg/mL rhuMAb CD20 in 25 mM

Tris and 50 mM sodium chloride at pH 6.0. The bulk was stored aseptically at 2-8°C in the absence of light.

Buffer exchange: The exchange of the bulk rhuMAb CD20 into different test buffers was done by dialysis at 2-8°C using Spectra/Por® 7 membranes (MWCO = 8 Kda) which were rinsed thoroughly with deionized water before use. One volume of antibody was dialyzed against a minimum of 10 volumes of the appropriate test buffer. This process was repeated three to four times within a one day period. To obtain the final concentration of 40 mg/mL, the antibody was further concentrated using an Amicon UF/DF cell containing a YM30 membrane (MWCO = 30 KDa). Due to the lower starting concentration of the 2.7 mg/mL material, this material was first concentrated to ~40 mg/mL and then dialyzed against the appropriate test buffer. After the target concentration was reached and the buffer exchange completed, trehalose, benzyl alcohol and polysorbate 20 were added to the final concentrations described in Tables 15 to 17 below. The liquid multidose C2B8 candidate formulation was 40 mg/mL rhuMAb CD20 formulated in 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0. The formulation was then sterile filtered through a 0.2μ membrane and the concentration was determined by UV spectrophotometric scan. A 0.5 ml of each final formulation was then filled into sterile 3 cc glass vials, stoppered with teflon faced grey butyl rubber stoppers and then capped with crimp seals.

Table 15: pH and buffer species comparison study

Concentration of rhuMAb CD20 (mg/mL)	Concentration of buffer species (mM)	pH	Storage Temperature (°C)
40	50 mM Acetate	5.0	2-8, 40, 50
40	50 mM Histidine	5.0	2-8, 40, 50
40	50 mM Histidine	6.0	2-8, 40, 50

All formulations contain 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20.

Table 16: Acetate buffering capacity study

Concentration of rhuMAb CD20 (mg/mL)	mM Acetate	Storage temperature (°C)
40	10	2-8, 40
40	15	2-8, 40
40	20	2-8, 40
40	25	2-8, 40
40	30	2-8, 40

All formulations contain 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0.

Table 17: Effective ratio of tonicity modifier : rhuMAb CD20 study

Tonicity modifier	Molar ratio of sugar/salt : rhuMAb CD20	mM trehalose or sodium chloride
Trehalose	0	0
Trehalose	250:1	67
Trehalose	500:1	134
Trehalose	1000:1	267
Sodium Chloride	500:1	134
Sodium Chloride	1000:1	267

A). All formulations contained 40 mg/mL rhuMAb CD20, 20 mM acetate, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0.

B). A freeze-thaw comparison study was completed for the trehalose formulations. The stability of all formulations at 2-8° and 40°C was conducted in parallel.

SEC HPLC: Samples were diluted to 10 mg/mL with formulation buffer before being assayed. The method uses a TSK G3000 SWXL column (TosoHaas) with a mobile phase consisting of 0.2M potassium phosphate, 0.25M potassium chloride, pH.7. The isocratic flow rate is 0.5 mL/min with a total run time of 30 minutes. The amount of protein injected is 200µg and the UV absorbance at 280 nm is used as the mode of detection.

HIC HPLC: Samples were diluted to 10 mg/mL with formulation buffer before being assayed. The antibody was digested with carboxypeptidase and papain prior to analysis of the final fragments. The method uses a TSK-GEL butyl-NPR (4.6 x 35 mm) column. The temperature of the column is controlled at 35°C during the assay. Elution of the antibody fragments is induced by changes in the ammonium sulfate gradient. The total run time of the assay is forty minutes and the flow rate is 1 ml/min. A protein load of 5-10µg is injected and the detection is monitored by UV absorbance at 214 nm.

UV spectrophotometric scan: For protein concentration determination, samples were accurately diluted 1:100 with formulation buffer. The absorbance at 280, 320 and 350 were read with an Hewlett Packard 8451A diode array spectrophotometer against the same formulation buffer as blank. The protein concentration was calculated by subtracting A_{320} from A_{280} and dividing by an extinction coefficient of 1.7.

For turbidity evaluation, samples were scanned without dilution on an Hewlett Packard 8451A diode array spectrophotometer and the average absorbance in the range of 340-360 nm was determined. Water was used as the blank.

Accelerated stability studies: The vials were stored upright in temperature-controlled rooms or incubators at 2-8°, 30°, 40° or 50°C. Two to three vials were removed at finite times and the protein degradation monitored by the stability indicating assays (SEC HPLC, HIC HPLC, UV spectrophotometric scan to determine protein concentration and turbidity, as well as pH measurement). In addition, samples were subjected to the complement dependent cell cytotoxicity assay as described below to assess bioactivity.

Freeze-thaw studies: Samples were exposed to a minimum of two hours of freezing at -70°C followed by a room temperature thaw (≤ 45 minutes). Each cycle was composed of one freeze

followed by one thaw excursion. Three vials were removed per formulation after one, three and five consecutive freeze-thaw cycles and the stability monitored by SEC HPLC and UV spectrophotometric scan to measure the turbidity and protein concentration.

Complement Dependent Cytotoxicity Assay (CDC): The bioactivity of the stability samples was determined by the CDC assay described in Gazzano Santoro *et al., J. Immunol. Meth.* 202:163-171 (1997), except that human complement (rather than rabbit complement) was used. The percent bioactivity of the test sample was determined as follows,

$$\% \text{ bioactivity} = \frac{[(\text{CDC assay mg/mL of sample}/\text{ protein concentration of sample})]}{[(\text{CDC assay mg/mL of reference}/\text{ protein concentration reference})]} \times 100$$

The protein concentration of the test sample and reference control were determined by UV spectrophotometric scan.

RESULTS AND DISCUSSION

pH and buffer species: Decreasing the pH from 7.5 to 5.0 of 40 mg/mL rhuMAb CD20 formulated in histidine and trehalose virtually eliminated oxidation of the antibody even after two months storage at 40°C (Figure 24B). There is also a decline in the rate of aggregation but the difference below pH 6.5 is slight (Figure 24A). Further reduction in aggregation rate may require a decrease in the protein concentration. A stable formulation appears to require a pH in the acidic range.

To differentiate between the effect of buffer species and the effect of pH, histidine (5 or 6) and acetate (5), multidose formulations were compared after storage at 2-8°, 40° or 50°C. The samples were stored at 50°C as a way of quickly determining the relative stability between the formulations with the caveat that the degradation observed was not necessarily predictive of that seen at 2-8°C storage.

The visual clarity of the formulations and the turbidity as measured by UV spectrophotometric scan (340-360 nm) after four (50°C) and eight (2-8°, 40°C) weeks storage is described in Table18. Both histidine formulations were more opalescent than the acetate at all temperatures studied. After two weeks storage at 50°C, the histidine at pH 5 had formed a solid opaque gel while the sibling formulation at pH 6 was visually cloudy and yellow in color by four weeks. The histidine formulations stored at 40°C also turned yellow. Without being bound to any one theory, the color formation is likely due to the oxidation of histidine and is more apparent in this study due to the high concentration of histidine used (50 mM). No differences were observed at 2-8°C storage relative to the initial timepoint.

Table 18: The effect of buffer species and pH on the appearance and clarity of 40 mg/mL rhuMAb CD20 multidose formulations containing 50 mM acetate or histidine, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5 or 6.

Formulation	Temperature	Appearance	Turbidity Avg. O.D. 340-360 nm
T = 8 weeks			
Acetate pH 5		Clear	0.051 ± 0.001
Histidine pH 5	2-8°C	Clear	0.065 ± 0.0002
Histidine pH 6		Clear	0.068 ± 0.001
Acetate pH 5	40°C	Opalescent	0.16 ± 0.002
Histidine pH 5	40°C	Light yellow, Opalescent	0.28 ± 0.015
Histidine pH 6	40°C	Light yellow, Opalescent	0.36 ± 0.026
T = 4 weeks			
Acetate pH 5	50°C	Very Opalescent	0.61 ± 0.007
Histidine pH 5	50°C	Firm opaque gel	N/D [1]
Histidine pH 6	50°C	Cloudy, yellow solution	1.30 ± 0.20

1. The turbidity was not determined (N/D) due to the gelation of the sample.

The stability was also monitored by HIC and SEC HPLC methods. After eight weeks storage at 40°C, the acetate pH 5 formulations were unchanged while the histidine pH 6 formulations had a 18 percent reduction in unoxidized Fc relative to the initial timepoint (Table 19). The percentage monomer decreased in all formulations stored at 40°C with the histidine pH 6 being slightly more stable. This reduction was attributed to the formation of a high molecular weight aggregate(s) eluting at the void volume, a lagging shoulder on the monomer peak and lower molecular weight species. The protein concentration and pH were also measured, except in the case of gelation, and no changes were observed over the duration of the study.

Table 19: The effect of buffer species and pH on the percentage unoxidized Fc and percentage monomer of 40 mg/mL rhuMAb CD20 multidose formulations containing 50 mM acetate or histidine, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5 or 6.

Formulation	Temperature	% Unoxidized Fc (HIC HPLC)	% Monomer (SEC HPLC)
T=0			
Acetate pH 5	2-8°C	93.9	99.4
Histidine pH 5	2-8°C	99.1	99.4
Histidine pH 6	2-8°C	99.3	98.5
T = 8 weeks			
Acetate pH 5	40°C	96.5	90.4
Histidine pH 5	40°C	87.0	87.4
Histidine pH 6	40°C	81.7	93.9

Since the acetate pH 5 formulation had only a slightly higher aggregation rate, did not turn yellow upon storage at high temperatures and had the greatest clarity under all conditions studied, it was chosen as the buffer species and pH of choice for all subsequent liquid C2B8 multidose formulation screens.

Amount of buffering species: The amount of acetate which maintained the pH of a 40 mg/mL C2B8 multidose formulation at 5.0 was determined. Summarized in Table 20 is the effect on pH as the acetate buffer concentration was increased from 10 to 30 mM. At two and four weeks there appeared to be a slight advantage in staying above 15 mM acetate, although this did not hold true upon long term storage. No change was seen in the pH in any of the test formulations studied after one years storage at 2-8°C. A range of 10 to 30 mM acetate is sufficient to maintain the pH at 5.0.

Table 20: The effect of acetate buffer concentration on maintaining the pH of liquid rhuMAb CD20 multidose formulations at 5.0. The formulations were composed of 40 mg/mL rhuMAb CD20, 10 to 30 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% polysorbate 20 at pH 5.0.

mM Acetate	pH (weeks) 0	2	4	56
2-8°C				
10	5.03	5.12	5.11	5.00
15	5.07	5.12	5.14	5.06
20	5.04	5.04	5.04	5.00
25	5.01	5.00	5.02	4.98
30	5.01	5.00	5.02	4.98
40°C				
10		5.12	5.14	
15		5.10	5.17	
20		5.08	5.10	
25		5.05	5.05	
30		5.03	5.05	

Ratio of tonicity modifier : protein: The addition of trehalose was beneficial to the stability of rhuMAb CD20 multidose formulations after multiple freeze/thaw cycles (Figure 25). A decrease in the formation of soluble aggregates was observed as the concentration of trehalose in the formulation was increased. At a ratio of 500 moles trehalose:1 mole C2B8 (134 mM trehalose), the percentage of aggregate formed was only ~0.5% after five freeze-thaw cycles while the control formulation containing no isotonicity modifier formed 4% aggregate. Having at least a 500 fold molar excess of trehalose in the liquid C2B8 formulation offered sufficient protection against freeze induced aggregation.

The ability of trehalose to stabilize rhuMAb CD20 during accelerated temperature storage was studied and compared to sodium chloride.

Figure 26 describes the effect of excess molar ratios of trehalose or sodium chloride on the clarity of test formulations stored at 40°C. Sodium chloride is deleterious to the stability. After two weeks storage at 40°C, the 500:1 ratio sample had an O.D. of 0.44. At a 1000:1 molar ratio, the sodium chloride containing formulation separated into a two phase system composed of an opaque gel covered with an opalescent fluid on top. In contrast, there was little change in the solution clarity of the samples containing 0 to 1000:1 molar ratio of trehalose even after one months storage at 40°C. Although the 2-8°C formulations were unchanged from the initial timepoint, the sodium chloride containing samples were more opalescent.

The effect of trehalose on minimizing the soluble aggregate formation was assessed by SEC HPLC (Figure 27). No differences were observed between the trehalose formulations and the negative control which contained no tonicity modifier. The formulations appeared to degrade at the same rate to the same products upon storage at 40°C. The pH and concentration were also maintained over the duration of the study .

The presence of trehalose is beneficial in minimizing freeze-induced aggregation and is not deleterious to the stability in the liquid state. The candidate liquid multidose formulation preferably contains at least 500:1 molar ratio of trehalose to C2B8.

Stability of candidate liquid multidose formulation: Based on the aforementioned studies, a protoype liquid multidose formulation composed of 40 mg/mL rhuMAb CD20, 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5.0 was placed on stability at 2-8°, 30°C and 40°C. The stability profile by SEC HPLC at each temperature studied is shown in Figure 28. Although the rate of aggregation is slightly faster at 40°C for the multidose formulation (40 mg/mL) compared to the reference control (10 mg/mL rhuMAb CD20, 25 mM citrate, 150 mM sodium chloride, 0.07% polysorbate 80 at pH 6.5), no decrease in percentage monomer was observed upon storage at 2-8°C for two years. The bioactivity of the two year old 2-8°C samples was 99.2% relative to the reference control as determined by the CDC assay.

CONCLUSIONS

The above screening studies indicated that a stable high concentration rhuMAb CD20 liquid multidose formulation was possible by buffering with acetate, maintaining the pH at 5 and including

preferably at least about 500 moles of trehalose per mole of antibody. The preferred liquid multidose configuration is composed of 40 mg/mL rhuMAb CD20, 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol and 0.02% polysorbate 20 at pH 5 and has a shelf life of two years at 2-8°C.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Genentech, Inc.

(ii) TITLE OF INVENTION: Antibody Formulation

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 1 DNA Way
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/874897
(B) FILING DATE: 13-JUN-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lee, Wendy M.
(B) REGISTRATION NUMBER: 40,378
(C) REFERENCE/DOCKET NUMBER: P1089R1PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650/225-1994
(B) TELEFAX: 650/952-9881

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 241 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr

20 25 30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
50 55 60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
65 70 75

Thr Ser Thr Ala Tyr Met Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
95 100 105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
110 115 120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
125 130 135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
140 145 150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
155 160 165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
170 175 180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
185 190 195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
200 205 210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
215 220 225

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
230 235 240

Leu
241

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 214 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Asn
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Gly Asn Thr Leu Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
110 115 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
125 130 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
140 145 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
155 160 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
170 175 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
185 190 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
200 205 210

Arg Gly Glu Cys
214

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Gly Gly Arg Met Lys Gln Leu Glu Asp Lys Val Glu Glu Leu
1 5 10 15

Leu Ser Lys Asn Tyr His Leu Glu Asn Glu Val Ala Arg Leu Lys
20 25 30

Lys Leu Val Gly Glu Arg
35 36

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Pro Lys Asn Ser Ser Met Ile Ser Asn Thr Pro
1 5 10 11

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

His Gln Ser Leu Gly Thr Gln
1 5 7

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

His Gln Asn Leu Ser Asp Gly Lys
1 5 8

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

His Gln Asn Ile Ser Asp Gly Lys
1 5 8

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Ile Ser Ser His Leu Gly Gln
1 5 8

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2143 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC 50

TCATTGCTGA GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC 100

TTCGCAATAT GGCGCAAAAT GACCAACAGC GGTTGATTGA TCAGGTAGAG 150

GGGGCGCTGT ACGAGGTAAA GCCCGATGCC AGCATTCCTG ACGACGATAC 200

GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT CCTCGTCAGT 250

AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT 300

TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG 350

CTCGCCGGGG ATCCTCTAGA GGTTGAGGTG ATTTTATGAA AAAGAATATC 400

GCATTTCTTC TTGCATCTAT GTTCGTTTTT TCTATTGCTA CAAACGCGTA 450

CGCTGATATC CAGATGACCC AGTCCCCGAG CTCCCTGTCC GCCTCTGTGG 500

GCGATAGGGT CACCATCACC TGTCGTGCCA GTCAGGACAT CAACAATTAT 550

CTGAACTGGT ATCAACAGAA ACCAGGAAAA GCTCCGAAAC TACTGATTTA 600

CTATACCTCC ACCCTCCACT CTGGAGTCCC TTCTCGCTTC TCTGGTTCTG 650

GTTCTGGGAC GGATTACACT CTGACCATCA GCAGTCTGCA ACCGGAGGAC 700

TTCGCAACTT ATTACTGTCA GCAAGGTAAT ACTCTGCCGC CGACGTTCGG 750

ACAGGGCACG AAGGTGGAGA TCAAACGAAC TGTGGCTGCA CCATCTGTCT 800

TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCCTCTGTT 850

GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA 900

GGTGGATAAC GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC 950

AGGACAGCAA GGACAGCACC TACAGCCTCA GCAGCACCCT GACGCTGAGC 1000

AAAGCAGACT ACGAGAAACA CAAAGTCTAC GCCTGCGAAG TCACCCATCA 1050

GGGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA GAGTGTTAAG 1100

CTGATCCTCT ACGCCGGACG CATCGTGGCG CTAGTACGCA AGTTCACGTA 1150

AAAACGGTAT CTAGAGGTTG AGGTGATTTT ATGAAAAAGA ATATCGCATT 1200

TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAC GCGTACGCTG 1250

AGGTTCAGCT GGTGGAGTCT GGCGGTGGCC TGGTGCAGCC AGGGGGCTCA 1300

CTCCGTTTGT CCTGTGCAAC TTCTGGCTAC ACCTTTACCG AATACACTAT 1350

GCACTGGATG CGTCAGGCCC CGGGTAAGGG CCTGGAATGG GTTGCAGGGA 1400

TTAATCCTAA AAACGGTGGT ACCAGCCACA ACCAGAGGTT CATGGACCGT 1450

TTCACTATAA GCGTAGATAA ATCCACCAGT ACAGCCTACA TGCAAATGAA 1500

CAGCCTGCGT GCTGAGGACA CTGCCGTCTA TTATTGTGCT AGATGGCGAG 1550

GCCTGAACTA CGGCTTTGAC GTCCGTTATT TTGACGTCTG GGGTCAAGGA 1600

ACCCTGGTCA CCGTCTCCTC GGCCTCCACC AAGGGCCCAT CGGTCTTCCC 1650

CCTGGCACCC TCCTCCAAGA GCACCTCTGG GGGCACAGCG GCCCTGGGCT 1700

GCCTGGTCAA GGACTACTTC CCCGAACCGG TGACGGTGTC GTGGAACTCA 1750

GGCGCCCTGA CCAGCGGCGT GCACACCTTC CCGGCTGTCC TACAGTCCTC 1800

AGGACTCTAC TCCCTCAGCA GCGTGGTGAC CGTGCCCTCC AGCAGCTTGG 1850

GCACCCAGAC CTACATCTGC AACGTGAATC ACAAGCCCAG CAACACCAAG 1900

GTCGACAAGA AAGTTGAGCC CAAATCTTGT GACAAAACTC ACACATGCCC 1950

GCCGTGCCCA GCACCAGAAC TGCTGGGCGG CCGCATGAAA CAGCTAGAGG 2000

ACAAGGTCGA AGAGCTACTC TCCAAGAACT ACCACCTAGA GAATGAAGTG 2050

GCAAGACTCA AAAAGCTTGT CGGGGAGCGC TAAGCATGCG ACGGCCCTAG 2100

AGTCCCTAAC GCTCGGTTGC CGCCGGGCGT TTTTTATTGT TAA 2143

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 237 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
-23         -20                 -15                 -10

Ser Ile Ala Thr Asn Ala Tyr Ala Asp Ile Gln Met Thr Gln Ser
              -5                   1               5

Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr
          10                  15                  20

Cys Arg Ala Ser Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln
          25                  30                  35

Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Thr Ser
          40                  45                  50

Thr Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser
          55                  60                  65

Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp
          70                  75                  80

Phe Ala Thr Tyr Tyr Cys Gln Gln Gly Asn Thr Leu Pro Pro Thr
          85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
         100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser
         115                 120                 125

Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg
         130                 135                 140

Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
         145                 150                 155

Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
         160                 165                 170
```

Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
175 180 185

Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser
190 195 200

Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
205 210 214

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 300 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
-23 -20 -15 -10

Ser Ile Ala Thr Asn Ala Tyr Ala Glu Val Gln Leu Val Glu Ser
-5 1 5

Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys
10 15 20

Ala Thr Ser Gly Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met
25 30 35

Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn
40 45 50

Pro Lys Asn Gly Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg
55 60 65

Phe Thr Ile Ser Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln
70 75 80

Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala
85 90 95

Arg Trp Arg Gly Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp
100 105 110

Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr
115 120 125

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr
130 135 140

Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe
145 150 155

```
Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
        160                 165                 170

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr
        175                 180                 185

Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr
        190                 195                 200

Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys
        205                 210                 215

Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
        220                 225                 230

Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Arg Met Lys
        235                 240                 245

Gln Leu Glu Asp Lys Val Glu Glu Leu Leu Ser Lys Asn Tyr His
        250                 255                 260

Leu Glu Asn Glu Val Ala Arg Leu Lys Lys Leu Val Gly Glu Arg
        265                 270                 275     277
```

WHAT IS CLAIMED IS:

1. A stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody not subjected to prior lyophilization, a buffer maintaining the pH in the range from about 4.5 to about 6.0, a surfactant and a polyol.

2. The formulation of claim 1 which is isotonic.

3. The formulation of claim 1 which is stable at a temperature of about 2-8°C for at least one year.

4. The formulation of claim 1 which is stable following freezing and thawing of the formulation.

5. The formulation of claim 1 which is stable at about 30°C for at least one month.

6. The formulation of claim 1 wherein the polyol is a nonreducing sugar.

7. The formulation of claim 6 wherein the nonreducing sugar is trehalose.

8. The formulation of claim 6 wherein the nonreducing sugar is sucrose.

9. The formulation of claim 1 wherein the antibody is an antibody fragment.

10. The formulation of claim 9 wherein the antibody fragment is a $F(ab')_2$.

11. The formulation of claim 1 wherein the antibody binds CD18.

12. The formulation of claim 1 wherein the buffer maintains the pH in the range from about 4.8 to about 5.5.

13. The formulation of claim 1 wherein the antibody concentration in the formulation is from about 0.1 to about 50 mg/mL.

14. The formulation of claim 1 wherein the surfactant is a polysorbate.

15. The formulation of claim 1 wherein the antibody binds CD20.

16. The formulation of claim 15 wherein the buffer is histidine or acetate.

17. The formulation of claim 16 wherein the histidine or acetate is present in an amount of about 5-30 mM.

18. The formulation of claim 15 further comprising a preservative.

19. The formulation of claim 18 wherein the preservative is benzyl alcohol.

20. The formulation of claim 15 wherein the antibody is present in an amount of about 30-50 mg/mL.

21. The formulation of claim 20 wherein the buffer is about 20-30 mM acetate at about pH 5, the polyol is trehalose in an amount of about 1-15% w/v, the surfactant is polysorbate in an amount of about 0.01-0.03%, and wherein the formulation further comprises benzyl alcohol in an amount of about 0.5 to 1%.

22. An article of manufacture comprising a container holding a stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody not subjected to prior lyophilization, a buffer maintaining the pH in the range from about 4.5 to about 6.0, a surfactant and a polyol.

23. A method for stabilizing an antibody in an aqueous pharmaceutical formulation by combining a therapeutically effective amount of an antibody not subjected to prior lyophilization, a buffer maintaining the pH in the range from about 4.5 to about 6.0, a surfactant and a polyol.

EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAGINPKNG
GTSHNQRFMDRFTISVDKSTSTAYMQMNSLRAEDTAVYYCARWRGLNYGFDVRYFD
VWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK
THTCPPCPAPELLGGRMKQLEDKVEELLSKNYHLENEVARLKKLVGER

FIG. 1A

DIQMTQSPSSLSASVGDRVTITCRASQDINNYLNWYQQKPGKAPKLLIYYTSTLHSGVP
SRFSGSGSGTDYTLTISSLQPEDFATYYCQQGNTLPPTFGQGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST
LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG. 1B

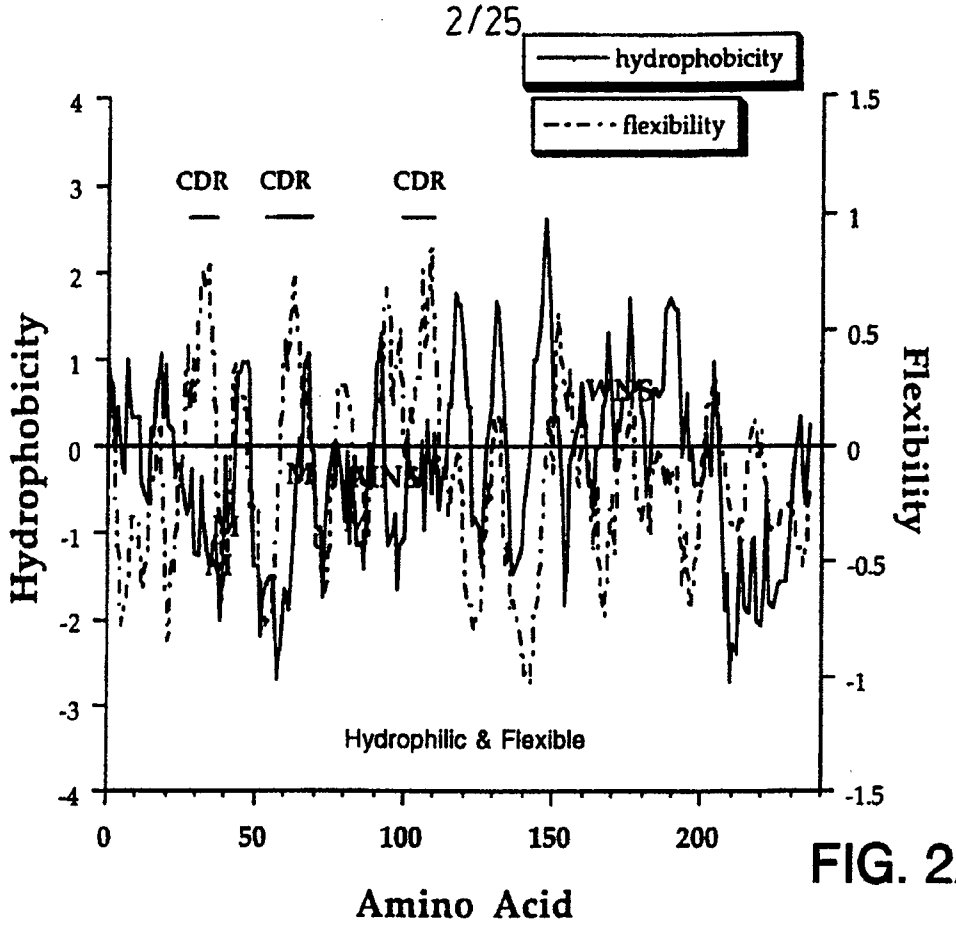

hydrophobicity
flexibility
CDR
CDR
CDR
Hydrophobicity
Flexibility
Hydrophilic & Flexible
Amino Acid

FIG. 2A

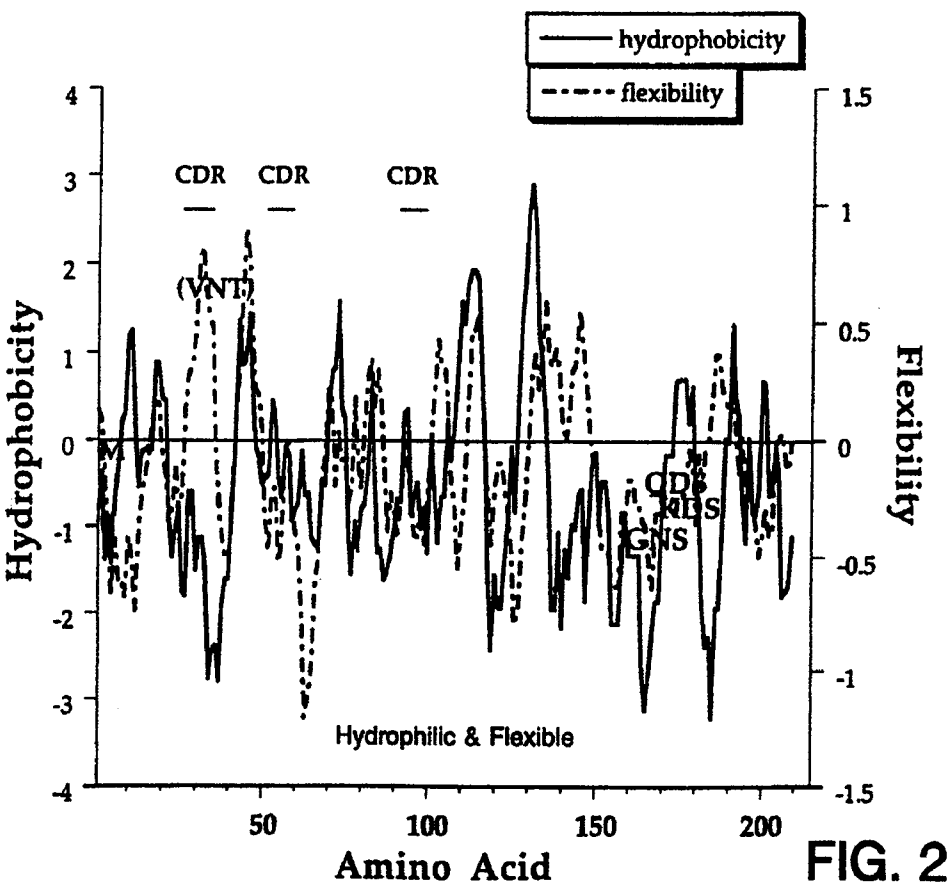

hydrophobicity
flexibility
CDR
CDR
CDR
Hydrophobicity
Flexibility
Hydrophilic & Flexible
Amino Acid

FIG. 2B

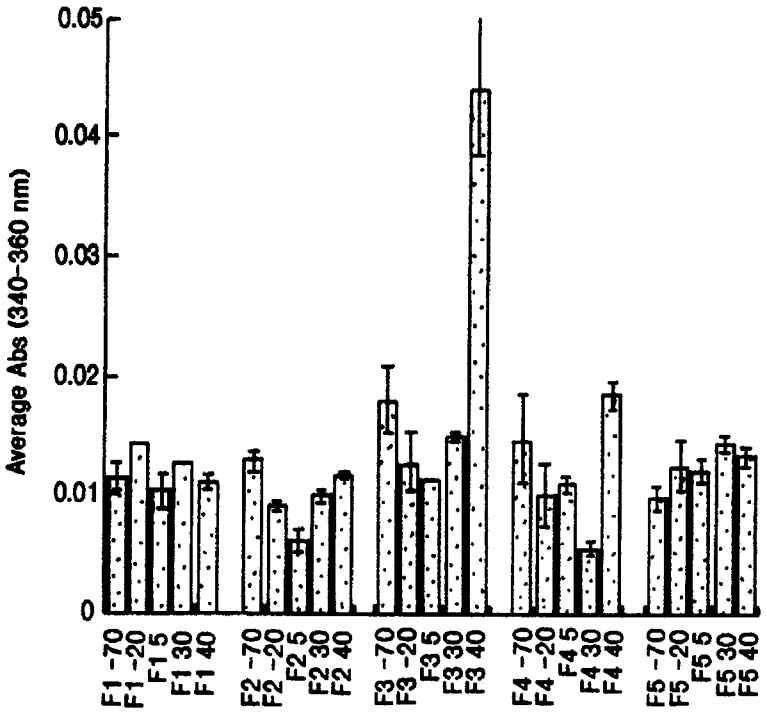

Average Abs (340-360 nm)
0.05
0.04
0.03
0.02
0.01
0
F1 -70
F1 -20
F1 5
F1 30
F1 40
F2 -70
F2 -20
F2 5
F2 30
F2 40
F3 -70
F3 -20
F3 5
F3 30
F3 40
F4 -70
F4 -20
F4 5
F4 30
F4 40
F5 -70
F5 -20
F5 5
F5 30
F5 40

FIG. 3A

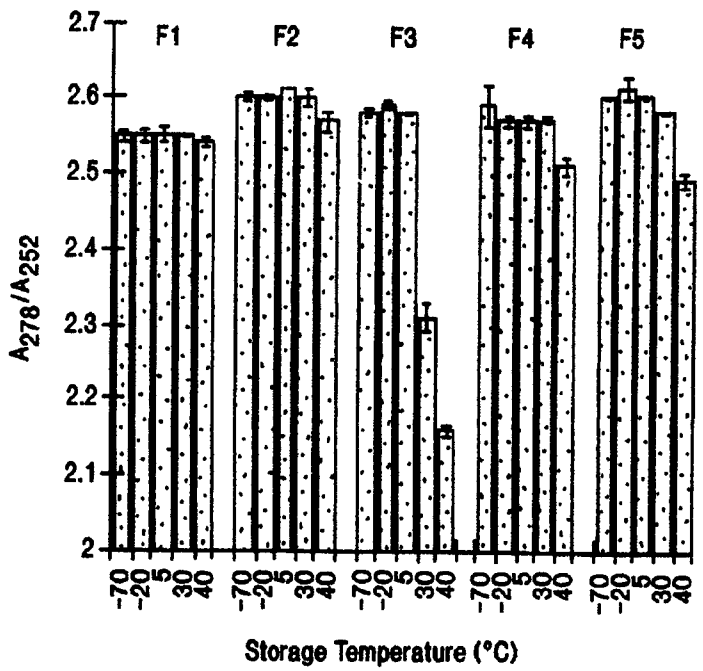

A_{278}/A_{252}
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2
F1
F2
F3
F4
F5
-70
-20
5
30
40
Storage Temperature (°C)

FIG. 3B

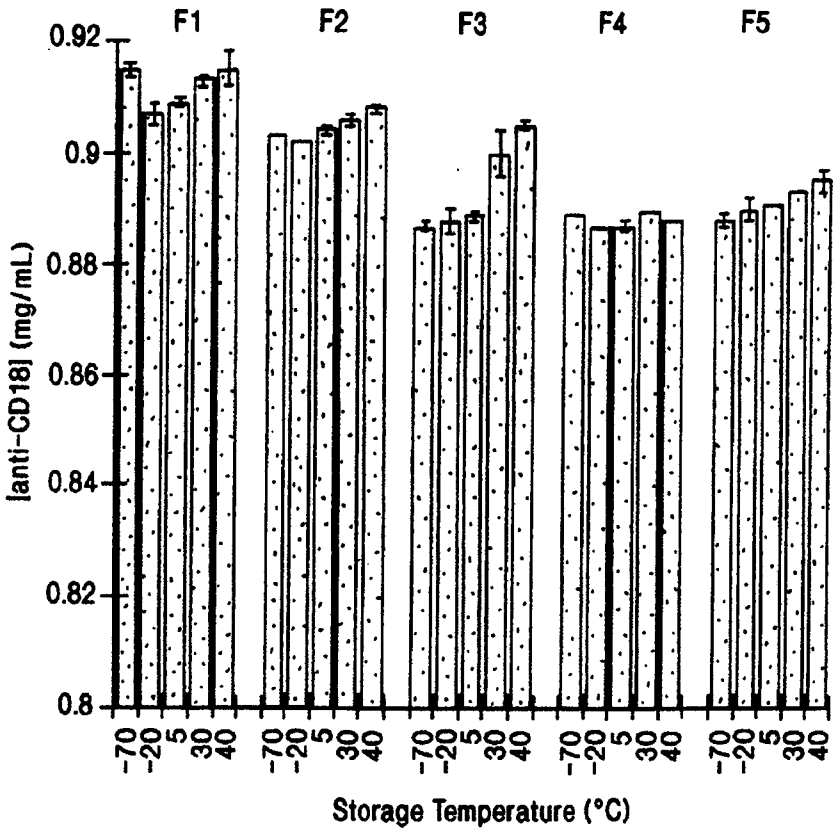

F1
F2
F3
F4
F5
0.92
0.9
0.88
0.86
0.84
0.82
0.8
[anti-CD18] (mg/mL)
-70
-20
5
30
40
Storage Temperature (°C)

FIG. 4

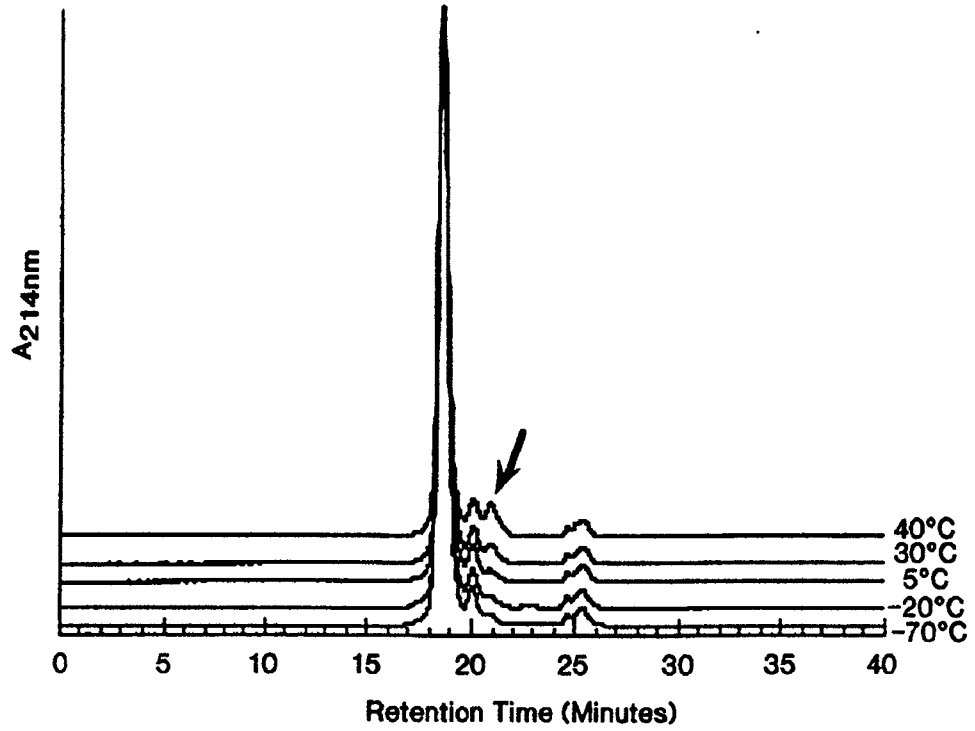

A214nm
40°C
30°C
5°C
-20°C
-70°C
0
5
10
15
20
25
30
35
40
Retention Time (Minutes)

FIG. 5A

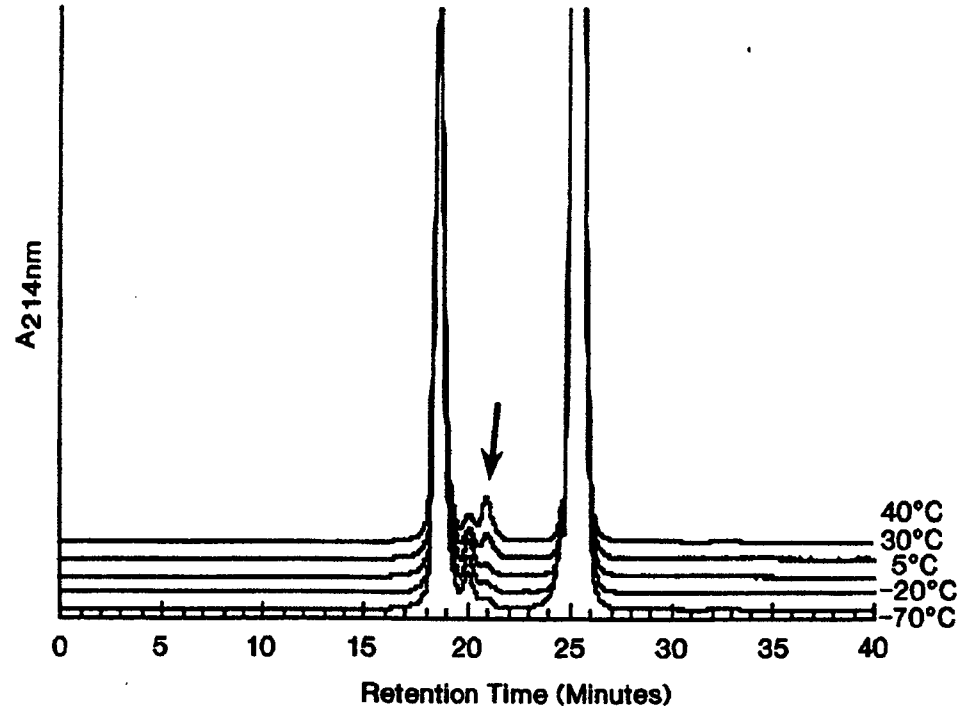

A214nm
40°C
30°C
5°C
-20°C
-70°C
0
5
10
15
20
25
30
35
40
Retention Time (Minutes)

FIG. 5B

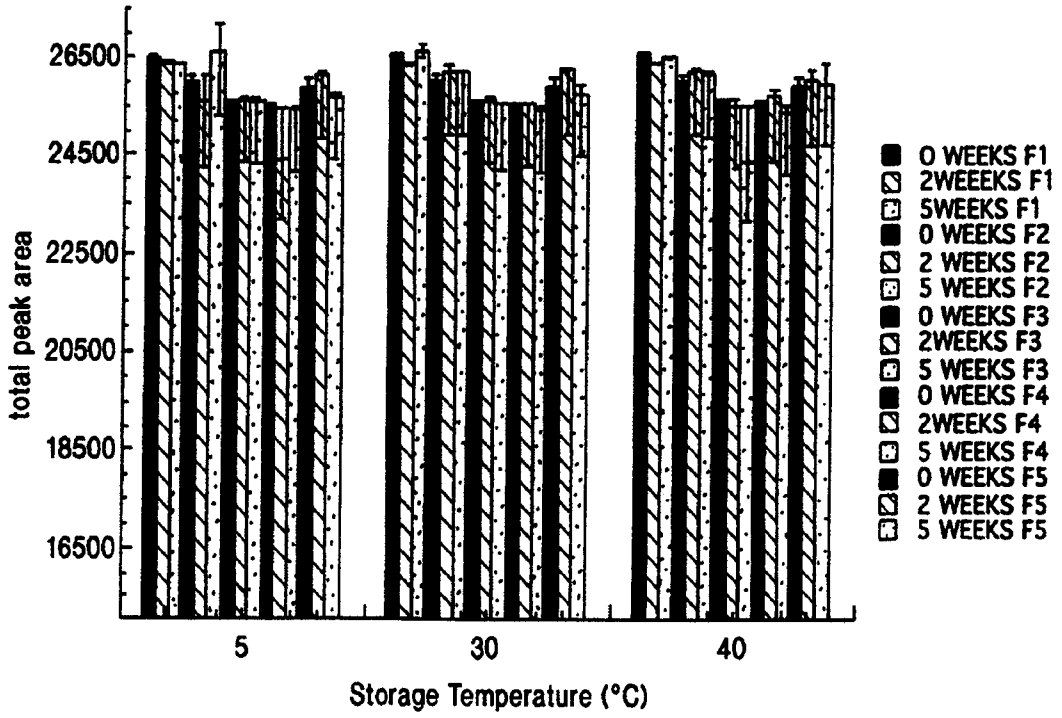

26500
24500
22500
20500
18500
16500
total peak area
5
30
40
Storage Temperature (°C)
0 WEEKS F1
2WEEEKS F1
5WEEKS F1
0 WEEKS F2
2 WEEKS F2
5 WEEKS F2
0 WEEKS F3
2WEEKS F3
5 WEEKS F3
0 WEEKS F4
2WEEKS F4
5 WEEKS F4
0 WEEKS F5
2 WEEKS F5
5 WEEKS F5

FIG. 6A

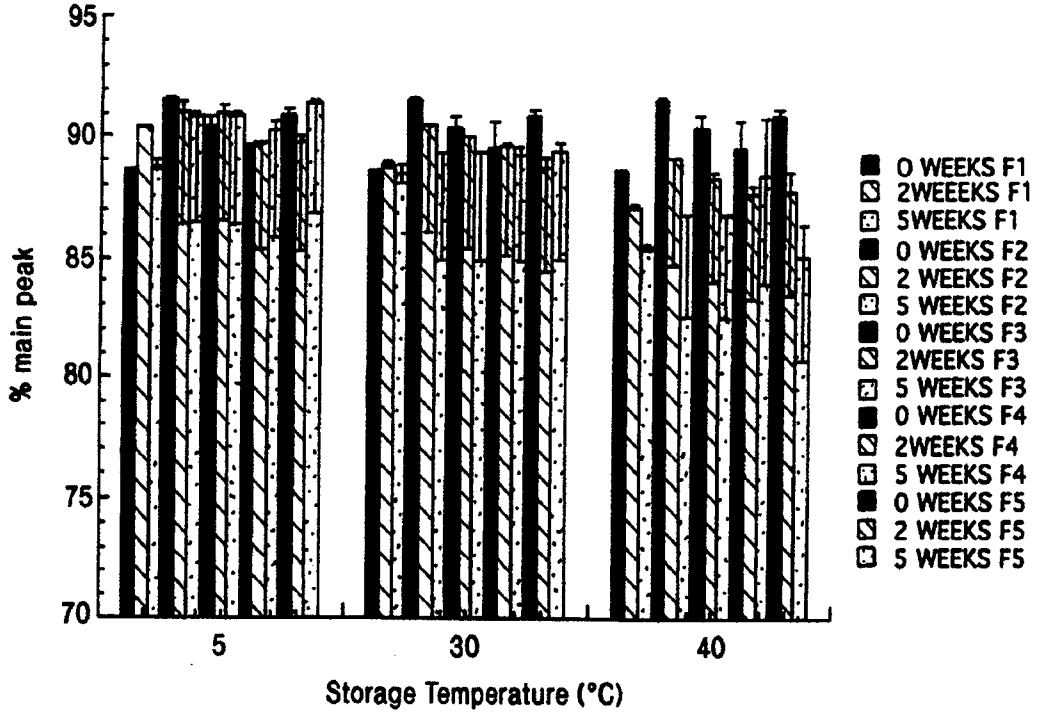

95
90
85
80
75
70
% main peak
5
30
40
Storage Temperature (°C)
0 WEEKS F1
2WEEEKS F1
5WEEKS F1
0 WEEKS F2
2 WEEKS F2
5 WEEKS F2
0 WEEKS F3
2WEEKS F3
5 WEEKS F3
0 WEEKS F4
2WEEKS F4
5 WEEKS F4
0 WEEKS F5
2 WEEKS F5
5 WEEKS F5

FIG. 6B

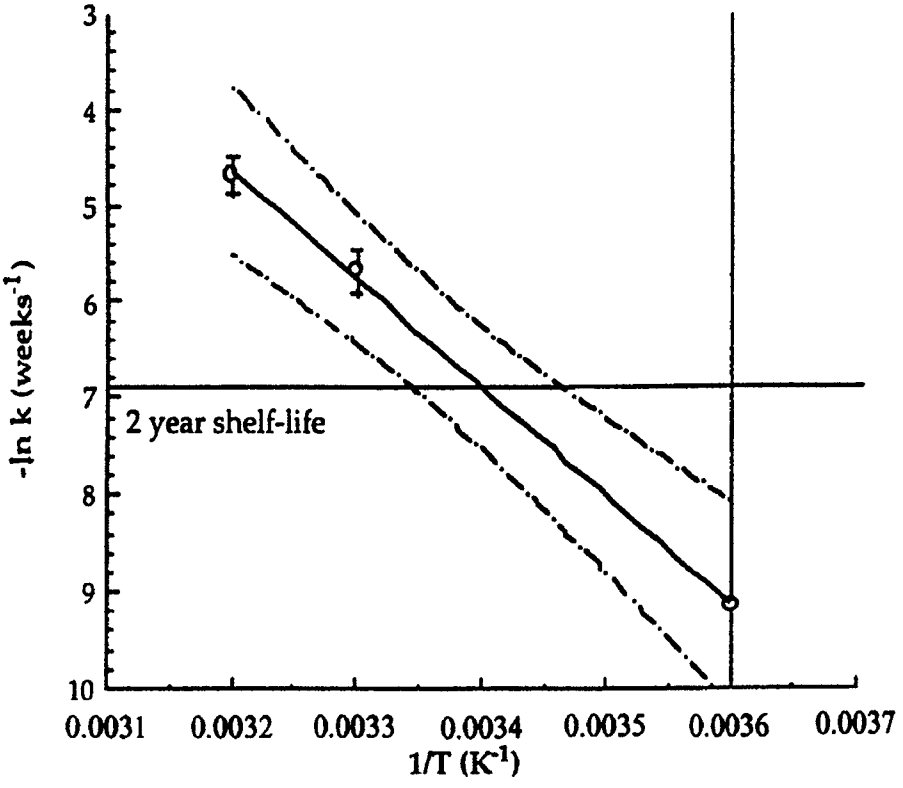

3
4
5
6
7
8
9
10
-ln k (weeks^{-1})
2 year shelf-life
0.0031
0.0032
0.0033
0.0034
0.0035
0.0036
0.0037
1/T (K^{-1})

FIG. 7

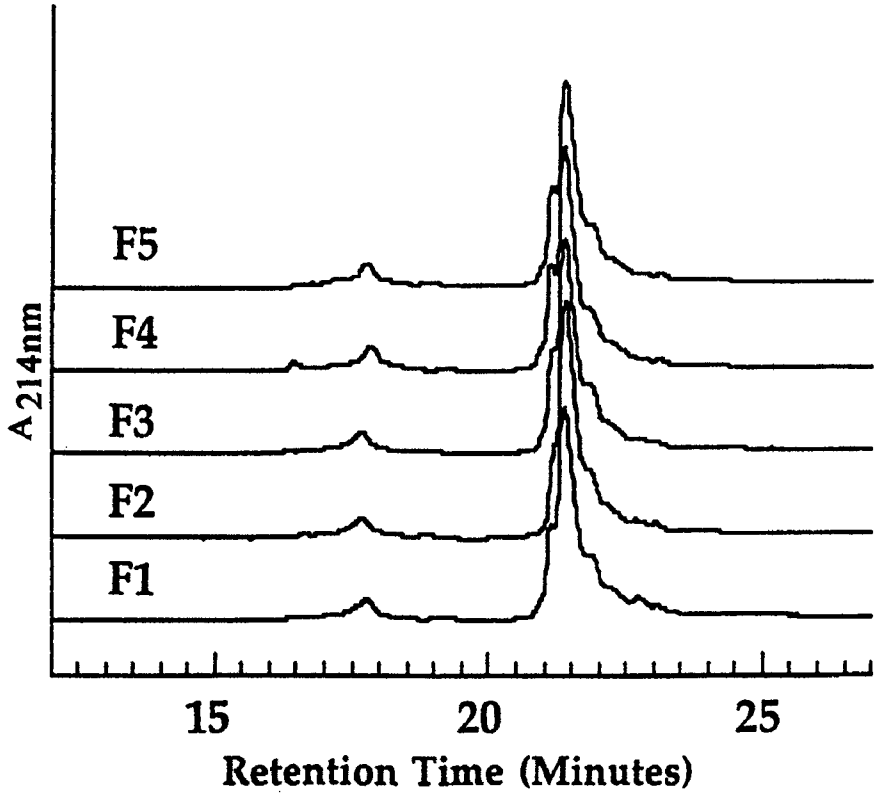

F5
F4
F3
F2
F1
A 214nm
15
20
25
Retention Time (Minutes)

FIG. 8

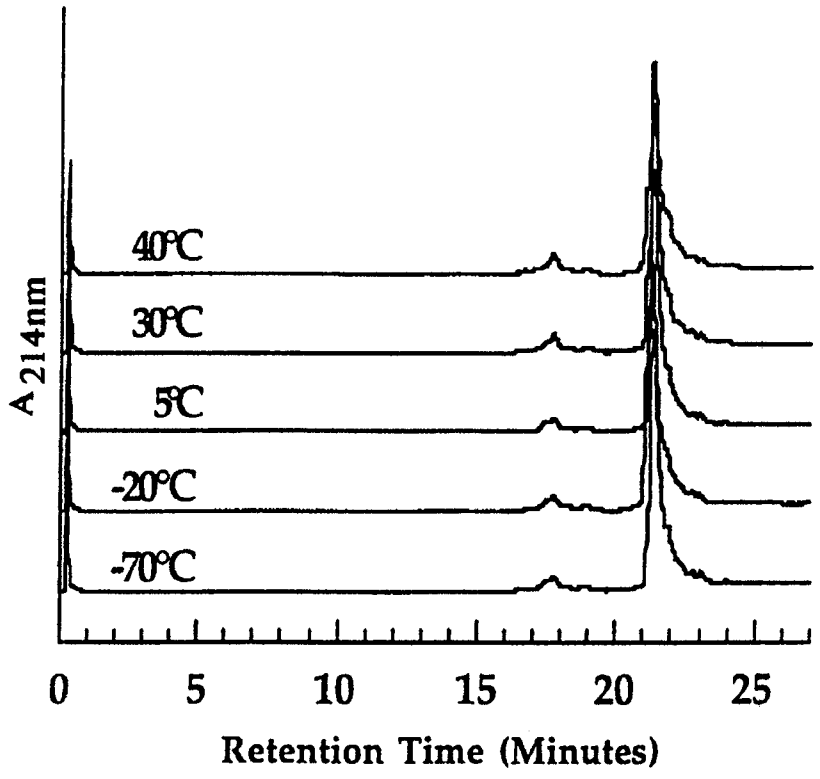

40°C
30°C
5°C
-20°C
-70°C
A214nm
0
5
10
15
20
25
Retention Time (Minutes)

FIG. 9

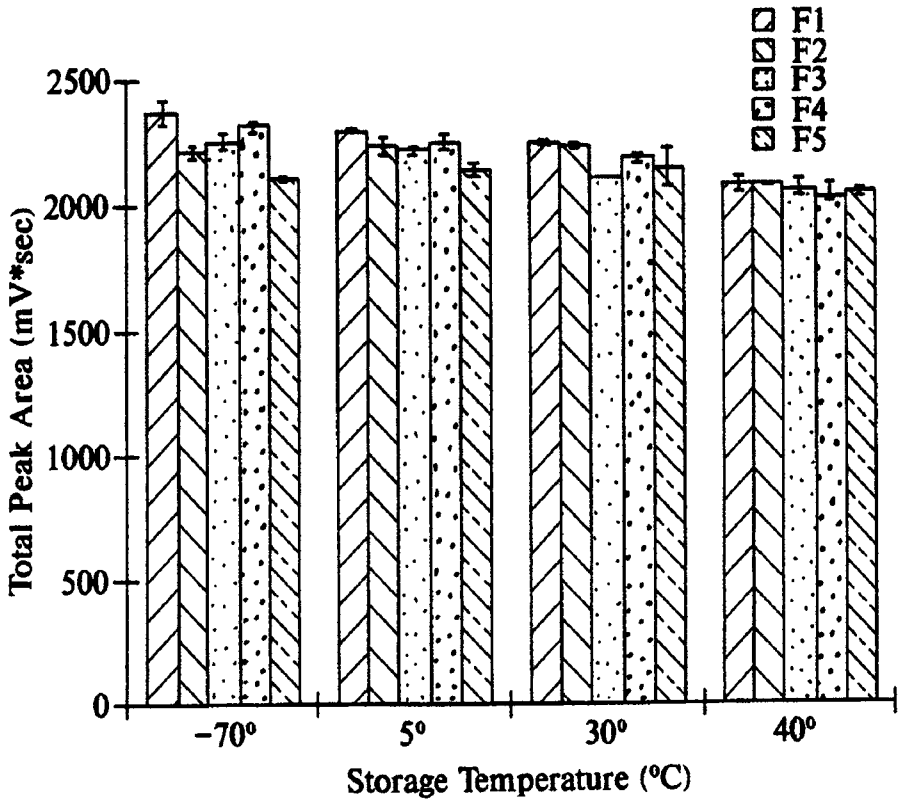

F1
F2
F3
F4
F5
2500
2000
1500
1000
500
0
Total Peak Area (mV*sec)
-70°
5°
30°
40°
Storage Temperature (°C)

FIG. 10

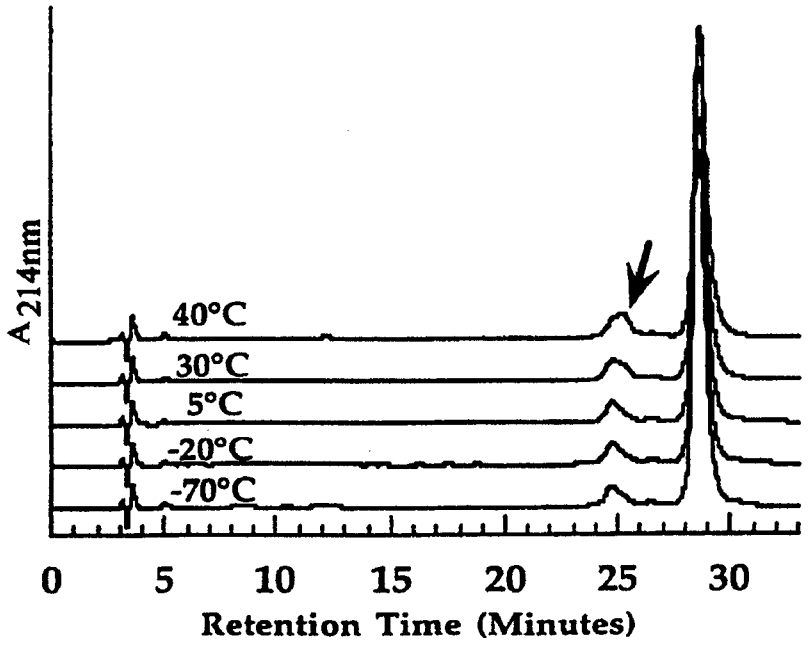

A 214nm
40°C
30°C
5°C
-20°C
-70°C
0
5
10
15
20
25
30
Retention Time (Minutes)

FIG. 11

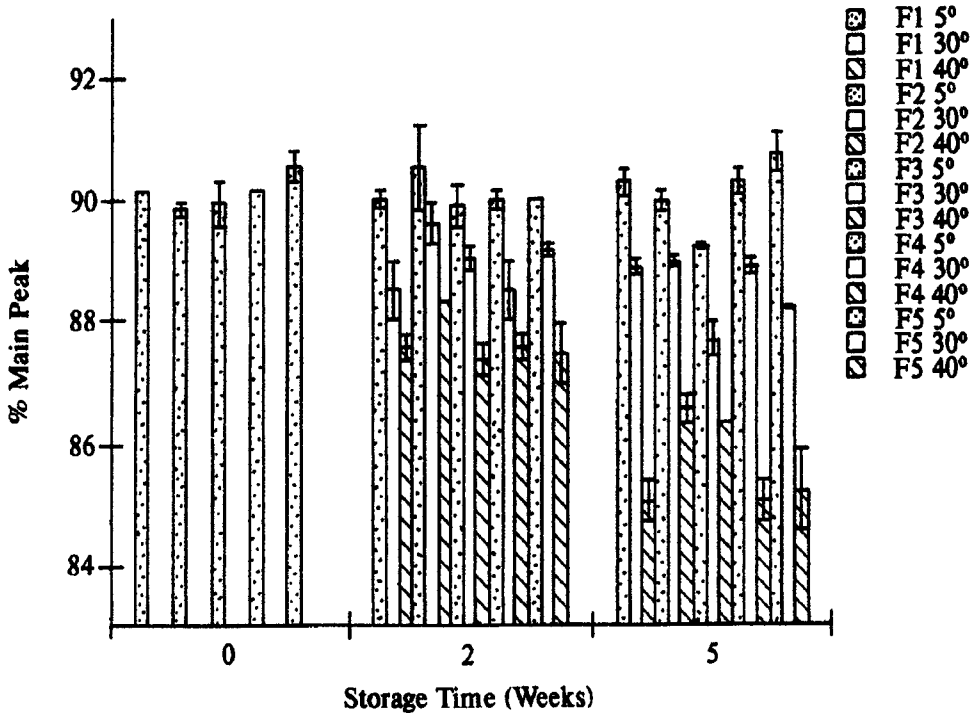

% Main Peak
92
90
88
86
84
0
2
5
Storage Time (Weeks)
F1 5°
F1 30°
F1 40°
F2 5°
F2 30°
F2 40°
F3 5°
F3 30°
F3 40°
F4 5°
F4 30°
F4 40°
F5 5°
F5 30°
F5 40°

FIG. 12

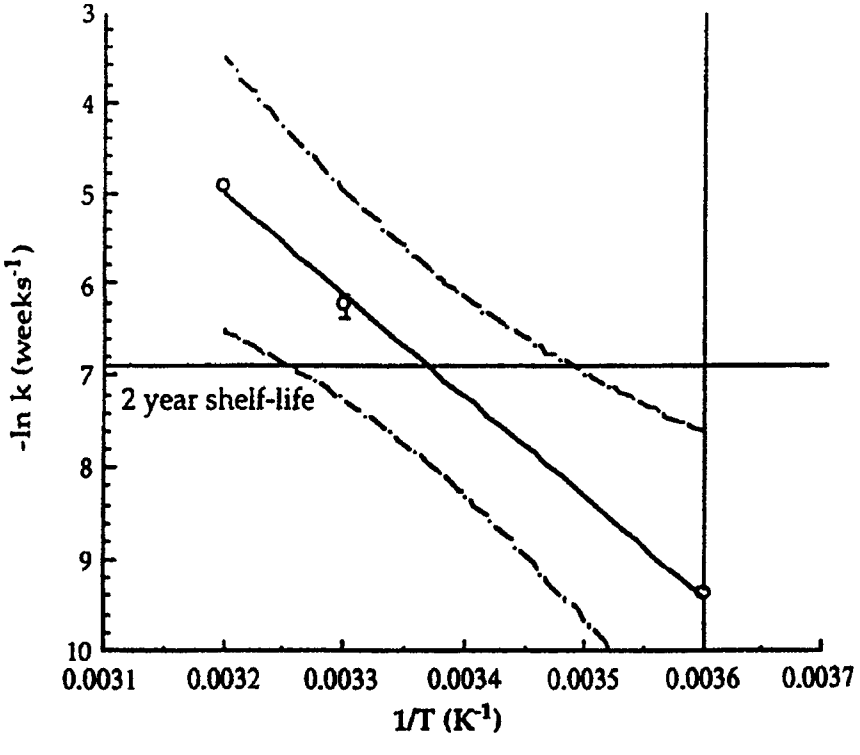

-ln k (weeks-1)
3
4
5
6
7
8
9
10
2 year shelf-life
0.0031
0.0032
0.0033
0.0034
0.0035
0.0036
0.0037
1/T (K-1)

FIG. 13

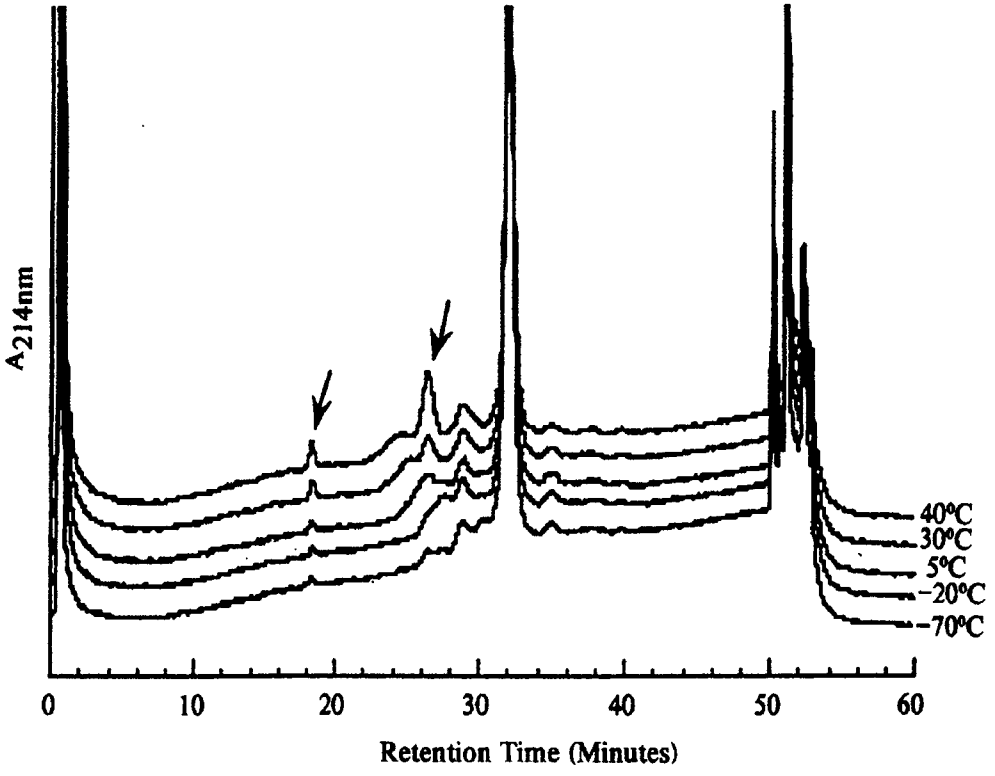


FIG. 14A

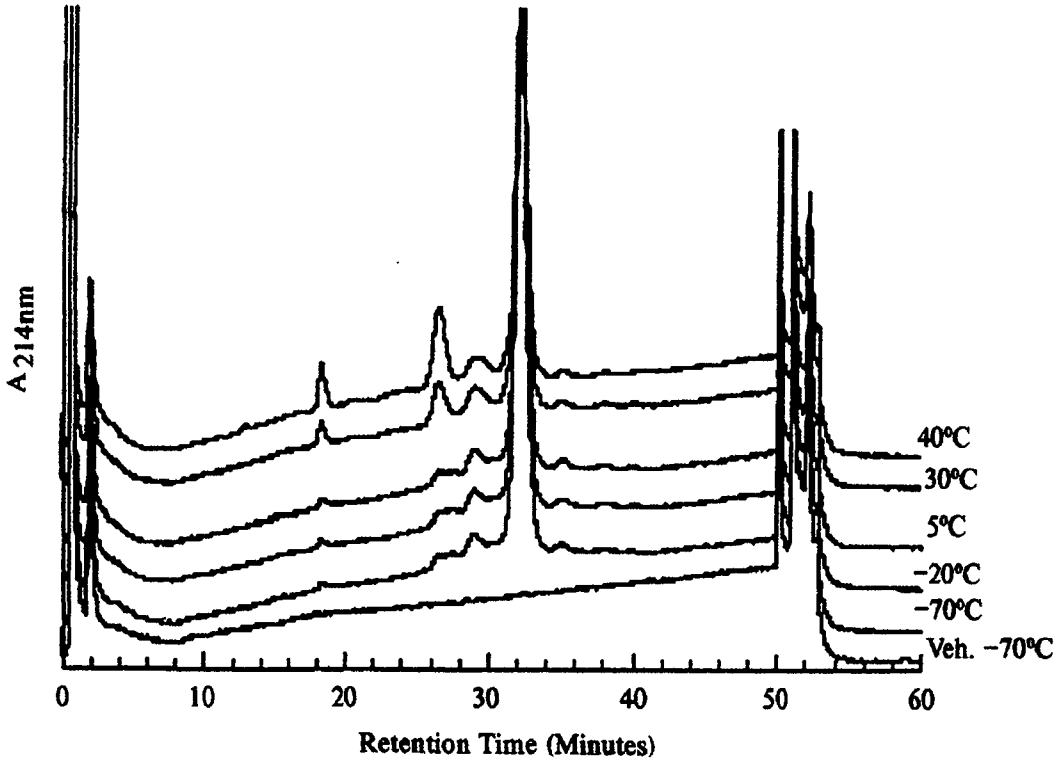


FIG. 14B

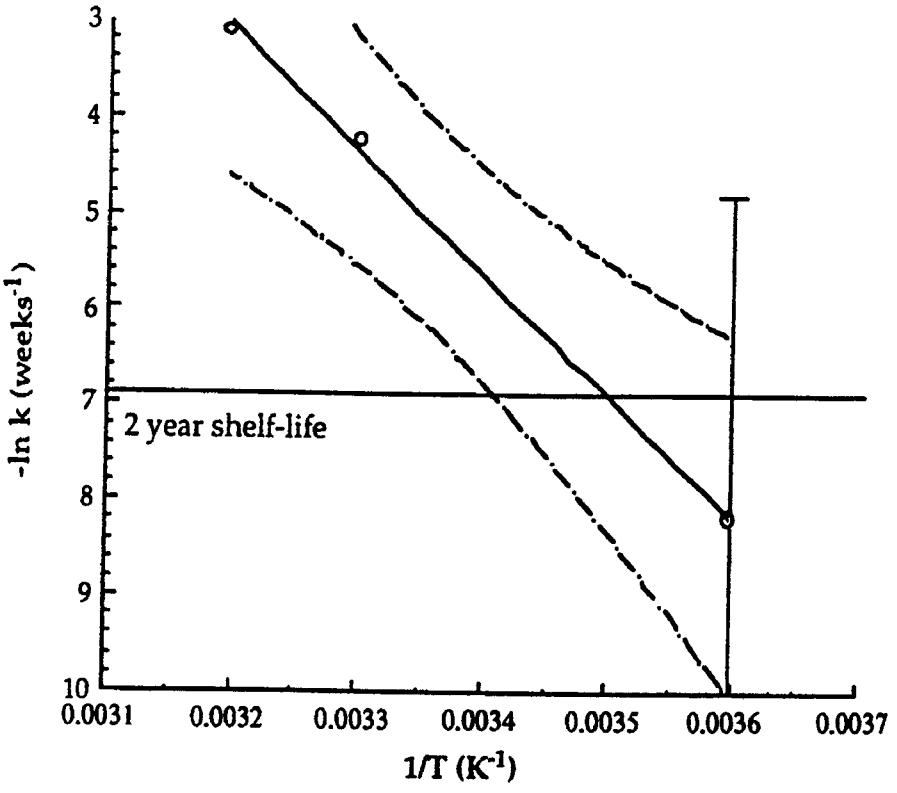

3
4
5
6
7
8
9
10
-ln k (weeks^{-1})
2 year shelf-life
0.0031
0.0032
0.0033
0.0034
0.0035
0.0036
0.0037
1/T (K^{-1})

FIG. 15

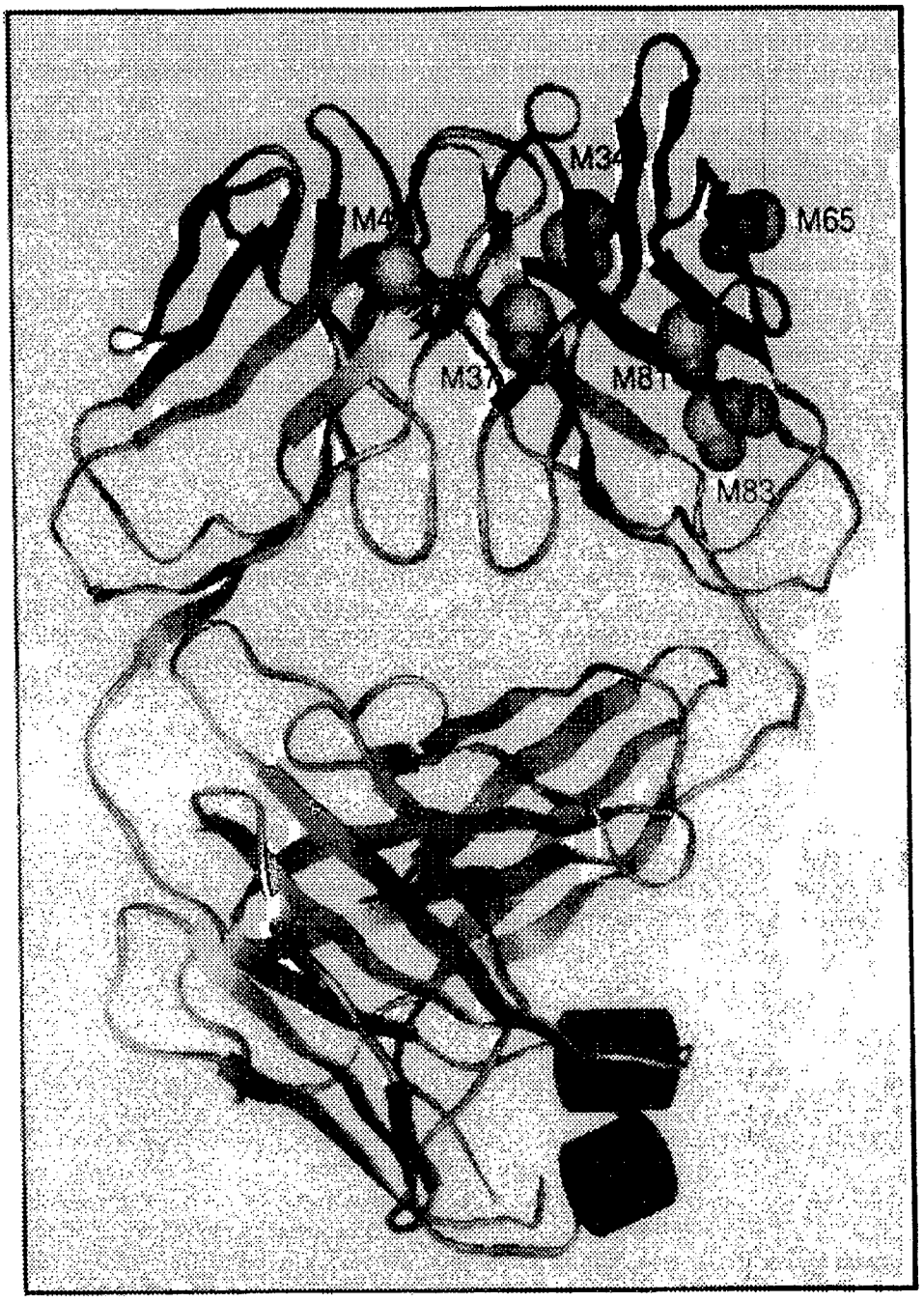


FIG. 16

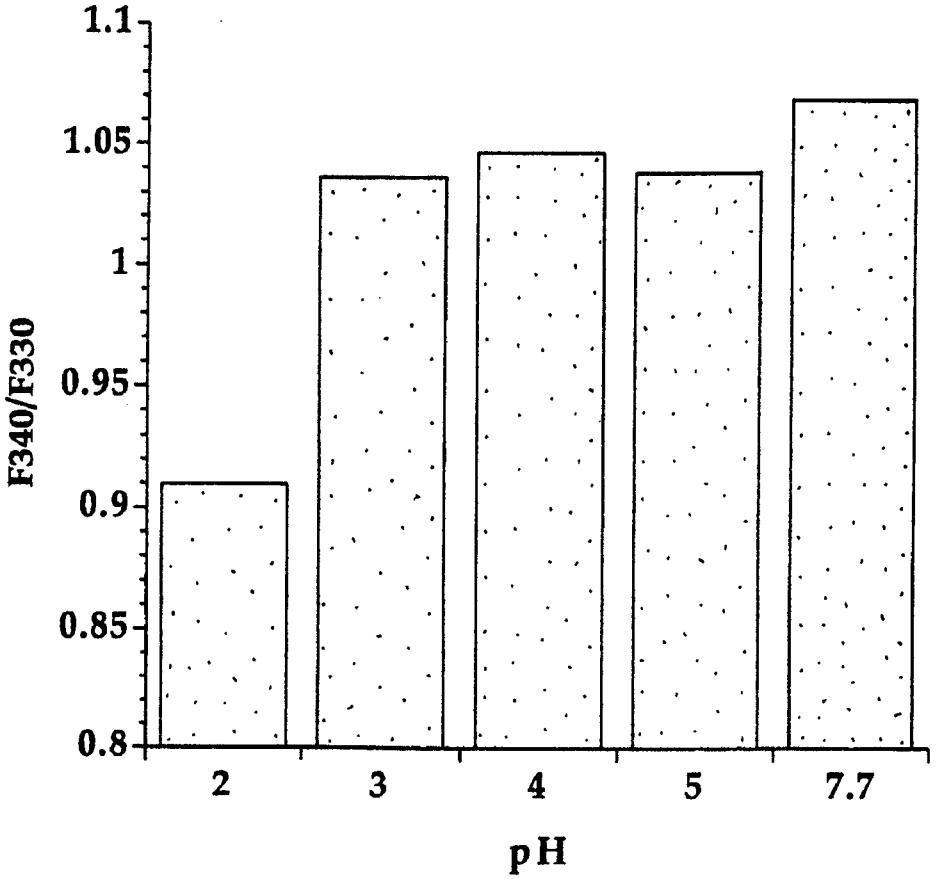


FIG. 17

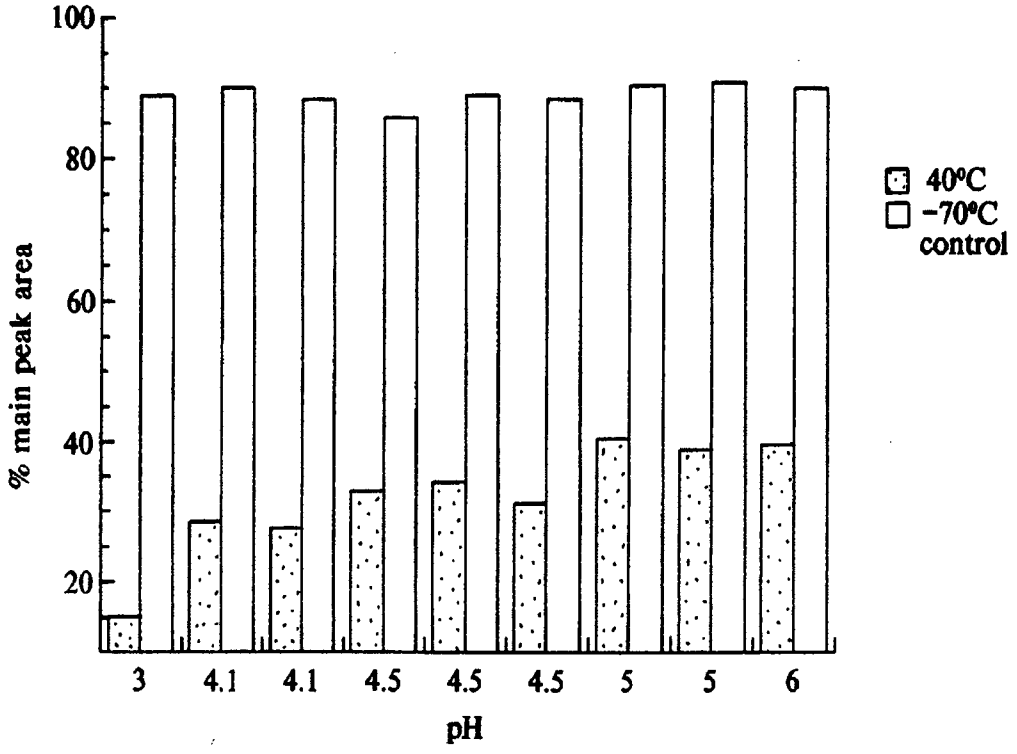


FIG. 18A

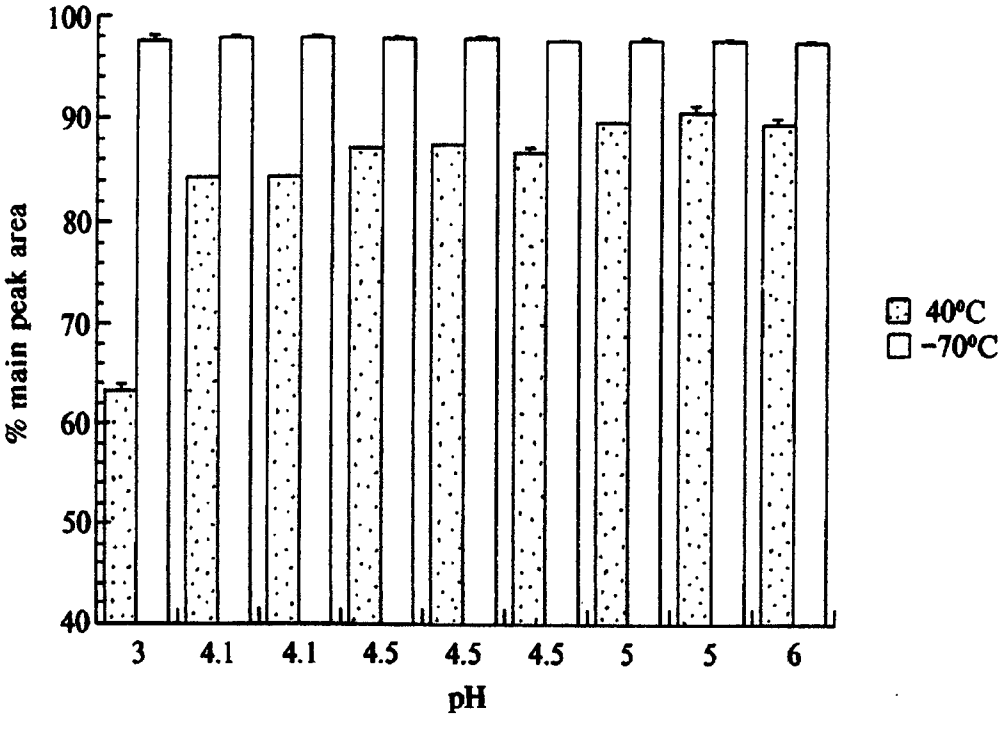


FIG. 18B

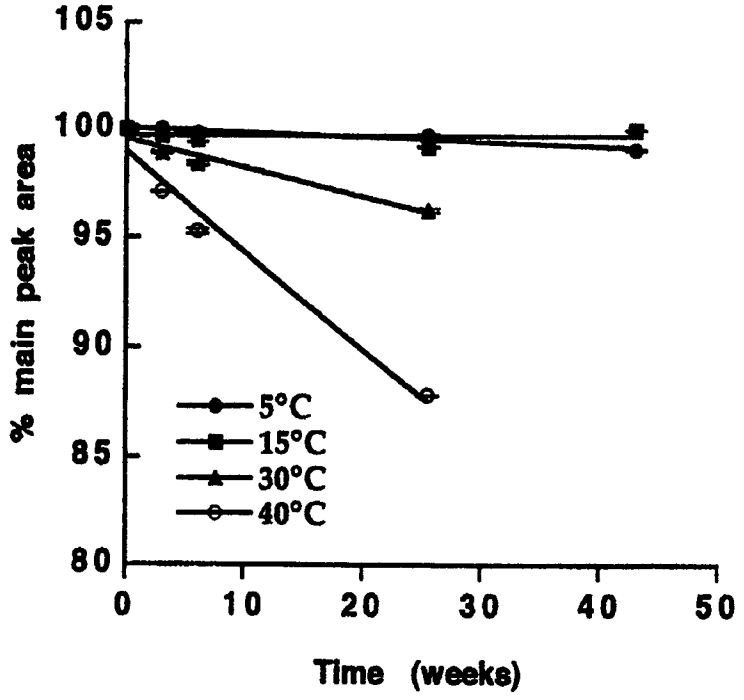


FIG. 19A

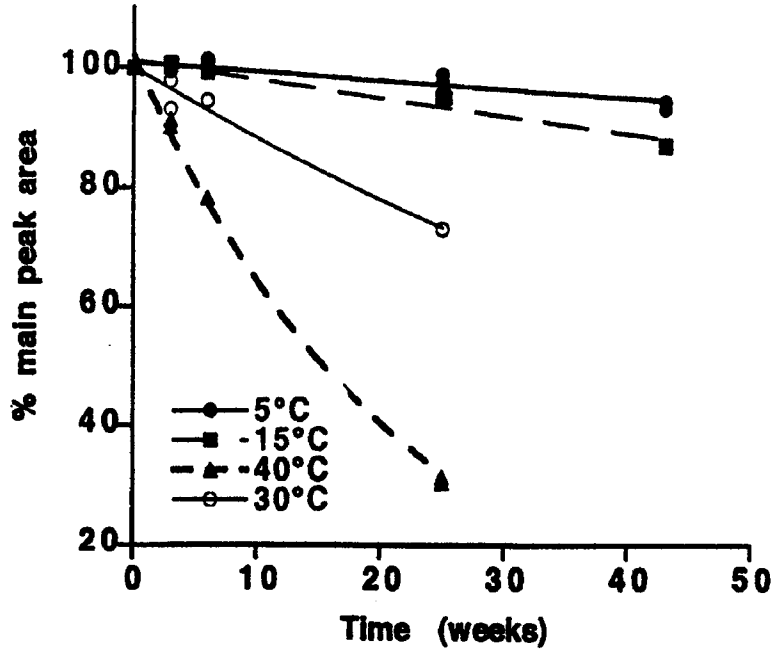


FIG. 19B

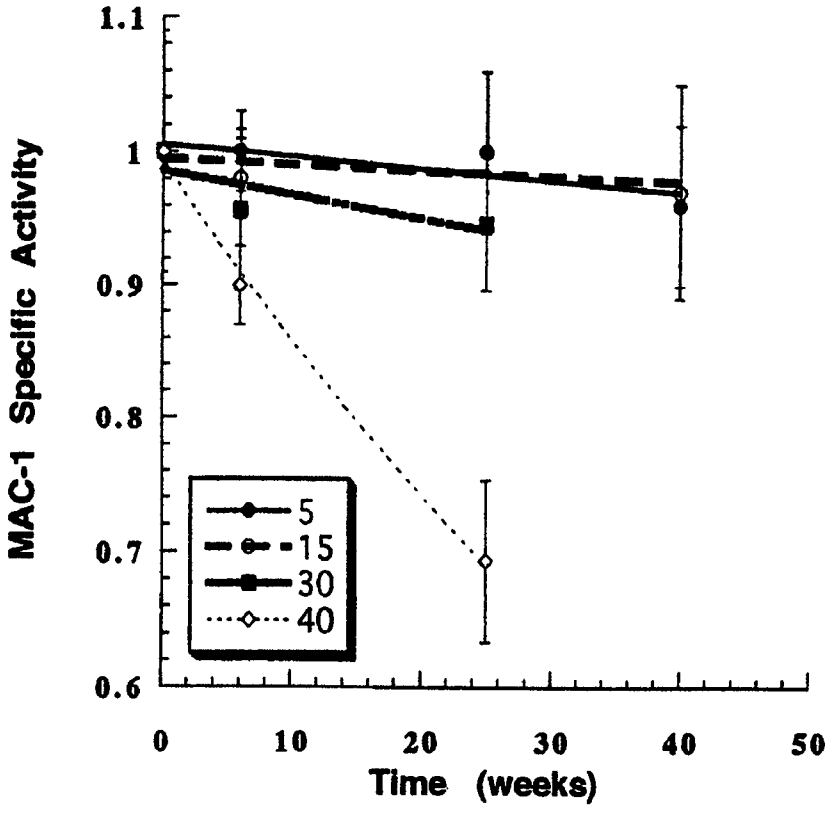

MAC-1 Specific Activity
1.1
1
0.9
0.8
0.7
0.6
0
10
20
30
40
50
Time (weeks)
5
15
30
40

FIG. 19C

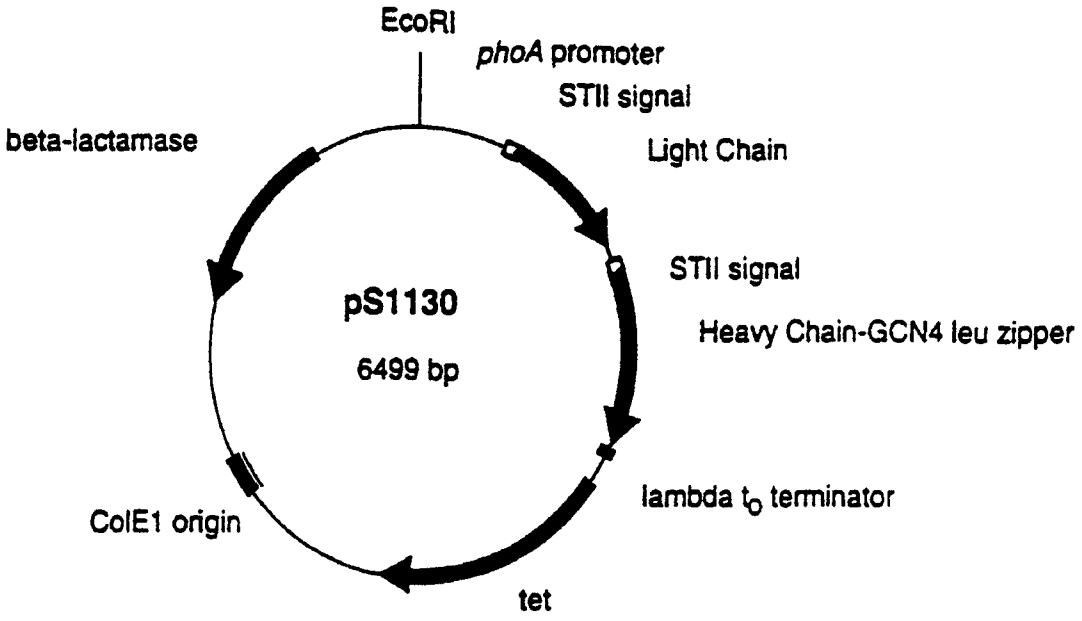

EcoRI
phoA promoter
STII signal
Light Chain
STII signal
Heavy Chain-GCN4 leu zipper
lambda t_0 terminator
tet
ColE1 origin
beta-lactamase
pS1130
6499 bp

FIG. 20

```
   1 GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA

  61 GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC TTCGCAATAT GGCGCAAAAT

 121 GACCAACAGC GGTTGATTGA TCAGGTAGAG GGGGCGCTGT ACGAGGTAAA GCCCGATGCC

 181 AGCATTCCTG ACGACGATAC GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT

 241 CCTCGTCAGT AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT

 301 TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG CTCGCCGGGG

 361 ATCCTCTAGA GGTTGAGGTG ATTTT ATG AAA AAG AAT ATC GCA TTT CTT CTT
 -23                           M   K   K   N   I   A   F   L   L

 413 GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAC GCT GAT ATC
 -14 A   S   M   F   V   F   S   I   A   T   N   A   Y   A   D   I

 461 CAG ATG ACC CAG TCC CCG AGC TCC CTG TCC GCC TCT GTG GGC GAT AGG
   3 Q   M   T   Q   S   P   S   S   L   S   A   S   V   G   D   R

 509 GTC ACC ATC ACC TGT CGT GCC AGT CAG GAC ATC AAC AAT TAT CTG AAC
  19 V   T   I   T   C   R   A   S   Q   D   I   N   N   Y   L   N

 557 TGG TAT CAA CAG AAA CCA GGA AAA GCT CCG AAA CTA CTG ATT TAC TAT
  35 W   Y   Q   Q   K   P   G   K   A   P   K   L   L   I   Y   Y

 605 ACC TCC ACC CTC CAC TCT GGA GTC CCT TCT CGC TTC TCT GGT TCT GGT
  51 T   S   T   L   H   S   G   V   P   S   R   F   S   G   S   G

 653 TCT GGG ACG GAT TAC ACT CTG ACC ATC AGC AGT CTG CAA CCG GAG GAC
  67 S   G   T   D   Y   T   L   T   I   S   S   L   Q   P   E   D

 701 TTC GCA ACT TAT TAC TGT CAG CAA GGT AAT ACT CTG CCG CCG ACG TTC
  83 F   A   T   Y   Y   C   Q   Q   G   N   T   L   P   P   T   F

 749 GGA CAG GGC ACG AAG GTG GAG ATC AAA CGA ACT GTG GCT GCA CCA TCT
  99 G   Q   G   T   K   V   E   I   K   R   T   V   A   A   P   S

 797 GTC TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA ACT GCC
 115 V   F   I   F   P   P   S   D   E   Q   L   K   S   G   T   A

 845 TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG GCC AAA GTA
 131 S   V   V   C   L   L   N   N   F   Y   P   R   E   A   K   V

 893 CAG TGG AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC TCC CAG GAG AGT
 147 Q   W   K   V   D   N   A   L   Q   S   G   N   S   Q   E   S

 941 GTC ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC
 163 V   T   E   Q   D   S   K   D   S   T   Y   S   L   S   S   T

 989 CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC TGC
 179 L   T   L   S   K   A   D   Y   E   K   H   K   V   Y   A   C

1037 GAA GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC TTC AAC
 195 E   V   T   H   Q   G   L   S   S   P   V   T   K   S   F   N

1085 AGG GGA GAG TGT TAA  G CTGATCCTCT ACGCCGGACG CATCGTGGCG
 211 R   G   E   C
```

FIG. 21A

```
1131 CTAGTACGCA AGTTCACGTA AAAACGGTAT CTAGAGGTTG AGGTGATTTT  ATG AAA
 -23                                                       M   K

1187 AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT
 -21 K   N   I   A   F   L   L   A   S   M   F   V   F   S   I   A

1235 ACA AAC GCG TAC GCT GAG GTT CAG CTG GTG GAG TCT GGC GGT GGC CTG
  -5 T   N   A   Y   A   E   V   Q   L   V   E   S   G   G   G   L

1283 GTG CAG CCA GGG GGC TCA CTC CGT TTG TCC TGT GCA ACT TCT GGC TAC
  12 V   Q   P   G   G   S   L   R   L   S   C   A   T   S   G   Y

1331 ACC TTT ACC GAA TAC ACT ATG CAC TGG ATG CGT CAG GCC CCG GGT AAG
  28 T   F   T   E   Y   T   M   H   W   M   R   Q   A   P   G   K

1379 GGC CTG GAA TGG GTT GCA GGG ATT AAT CCT AAA AAC GGT GGT ACC AGC
  44 G   L   E   W   V   A   G   I   N   P   K   N   G   G   T   S

1427 CAC AAC CAG AGG TTC ATG GAC CGT TTC ACT ATA AGC GTA GAT AAA TCC
  60 H   N   Q   R   F   M   D   R   F   T   I   S   V   D   K   S

1475 ACC AGT ACA GCC TAC ATG CAA ATG AAC AGC CTG CGT GCT GAG GAC ACT
  76 T   S   T   A   Y   M   Q   M   N   S   L   R   A   E   D   T

1523 GCC GTC TAT TAT TGT GCT AGA TGG CGA GGC CTG AAC TAC GGC TTT GAC
  92 A   V   Y   Y   C   A   R   W   R   G   L   N   Y   G   F   D

1571 GTC CGT TAT TTT GAC GTC TGG GGT CAA GGA ACC CTG GTC ACC GTC TCC
 108 V   R   Y   F   D   V   W   G   Q   G   T   L   V   T   V   S

1619 TCG GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC TCC TCC
 124 S   A   S   T   K   G   P   S   V   F   P   L   A   P   S   S

1667 AAG AGC ACC TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC AAG GAC
 140 K   S   T   S   G   G   T   A   A   L   G   C   L   V   K   D

1715 TAC TTC CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA GGC GCC CTG ACC
 156 Y   F   P   E   P   V   T   V   S   W   N   S   G   A   L   T

1763 AGC GGC GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC TCA GGA CTC TAC
 172 S   G   V   H   T   F   P   A   V   L   Q   S   S   G   L   Y

1811 TCC CTC AGC AGC GTG GTG ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG
 188 S   L   S   S   V   V   T   V   P   S   S   S   L   G   T   Q

1859 ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC AAC ACC AAG GTC GAC
 204 T   Y   I   C   N   V   N   H   K   P   S   N   T   K   V   D

1907 AAG AAA GTT GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCG CCG
 220 K   K   V   E   P   K   S   C   D   K   T   H   T   C   P   P

1955 TGC CCA GCA CCA GAA CTG CTG GGC GGC CGC ATG AAA CAG CTA GAG GAC
 236 C   P   A   P   E   L   L   G   G   R   M   K   Q   L   E   D

2003 AAG GTC GAA GAG CTA CTC TCC AAG AAC TAC CAC CTA GAG AAT GAA GTG
 252 K   V   E   E   L   L   S   K   N   Y   H   L   E   N   E   V

2051 GCA AGA CTC AAA AAG CTT GTC GGG GAG CGC TAA  GCATGCG ACGGCCCTAG
 268 A   R   L   K   K   L   V   G   E   R

2101 AGTCCCTAAC GCTCGGTTGC CGCCGGGCGT TTTTTATTGT TAA
```

FIG. 21B

Strain	Genotype
W3110	K-12 F⁻ lambda⁻ IN*rmD-rmE1*
↓	
1A2	W3110 Δ*fhuA*
↓	
7C1	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*)
↓	
16C9	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC*
↓	
23E3	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC* Δ*degP*
↓	
33B6	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC* Δ*degP ilvG*
↓	
49B2	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC* Δ*degP ilvG* Δ*fucP*
↓	
49A5	W3110 ΔfhuA Δpho A Δ(*argF-lac*) *deoC* ΔdegP *ilvG* ΔfucP ΔmalE

FIG. 22

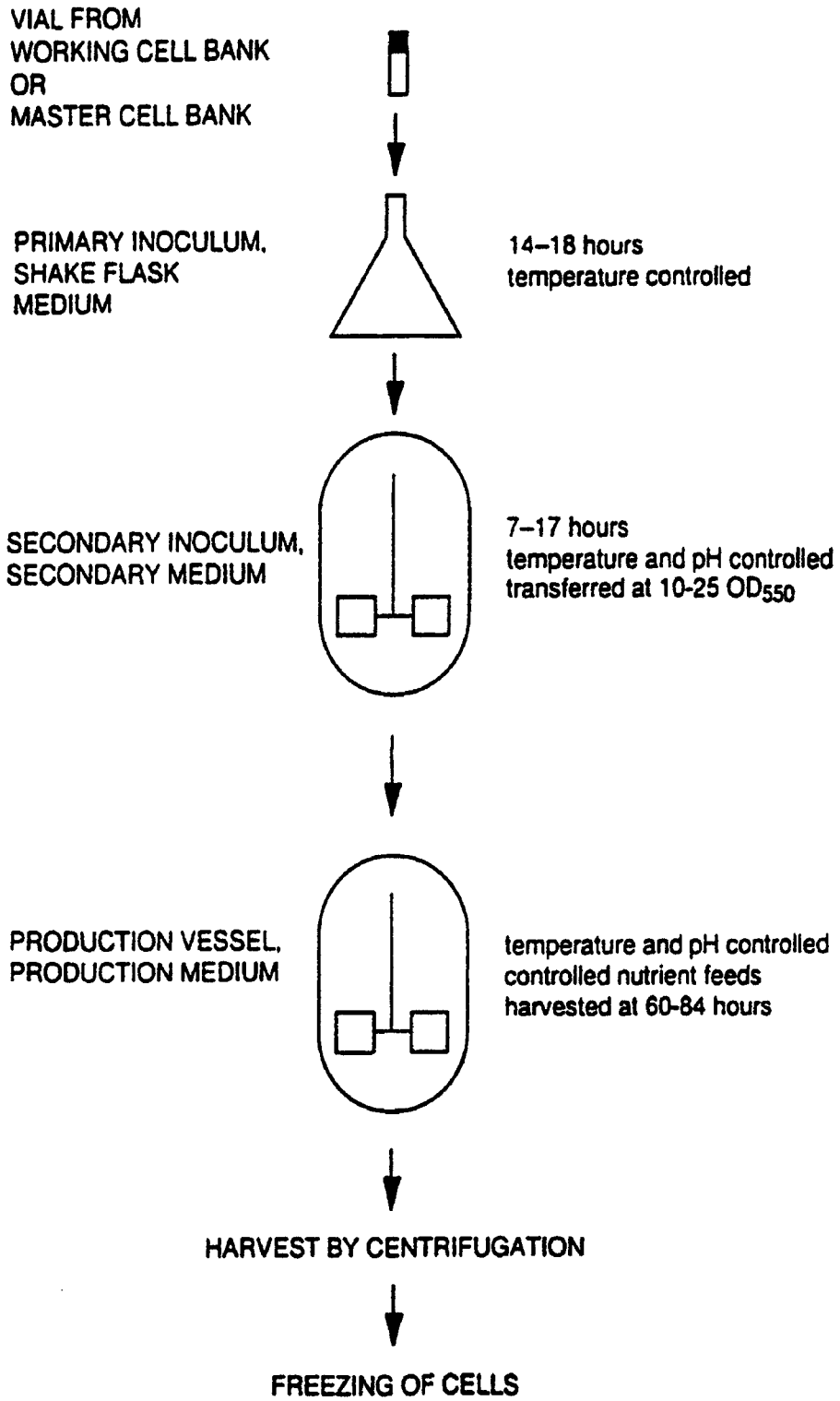

VIAL FROM
WORKING CELL BANK
OR
MASTER CELL BANK
PRIMARY INOCULUM,
SHAKE FLASK
MEDIUM
14–18 hours
temperature controlled
SECONDARY INOCULUM,
SECONDARY MEDIUM
7–17 hours
temperature and pH controlled
transferred at 10-25 OD550
PRODUCTION VESSEL,
PRODUCTION MEDIUM
temperature and pH controlled
controlled nutrient feeds
harvested at 60-84 hours
HARVEST BY CENTRIFUGATION
FREEZING OF CELLS

FIG. 23

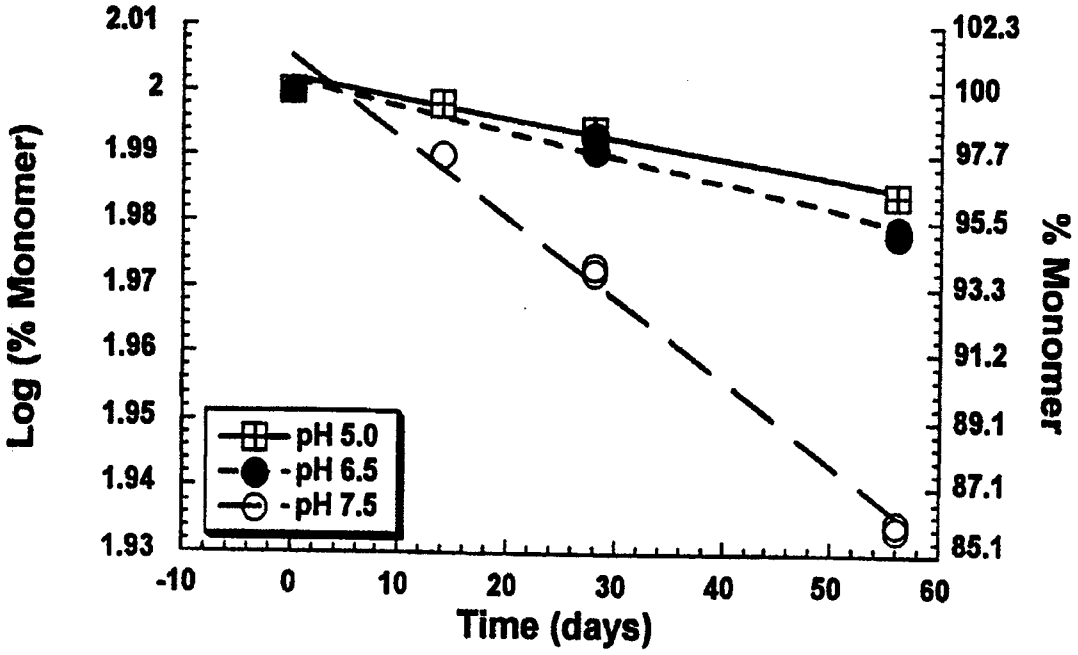


FIG. 24A

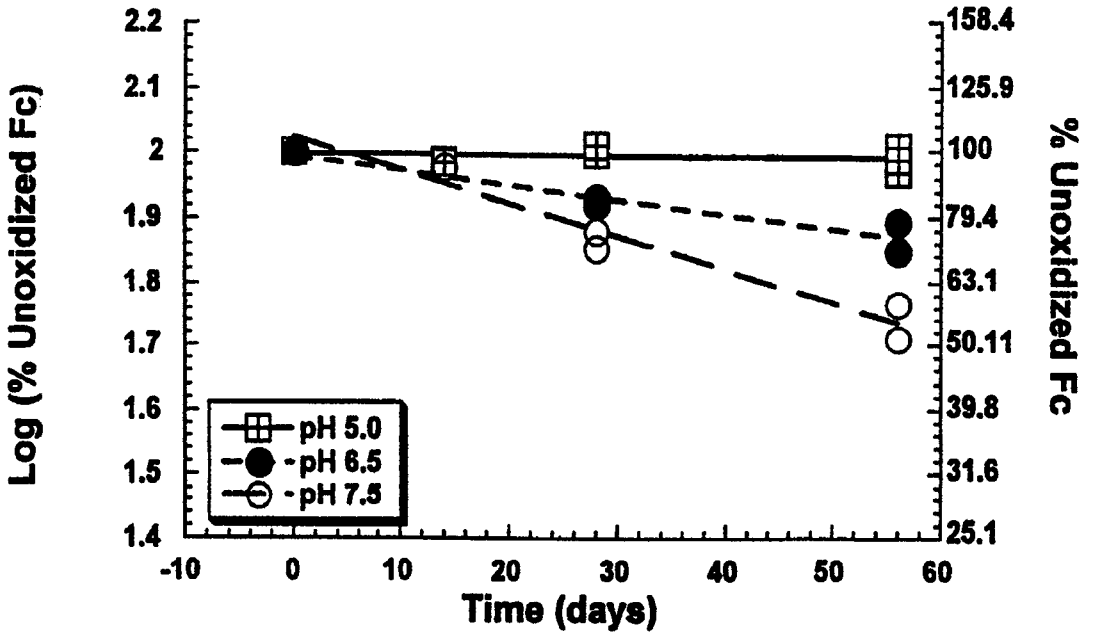


FIG. 24B

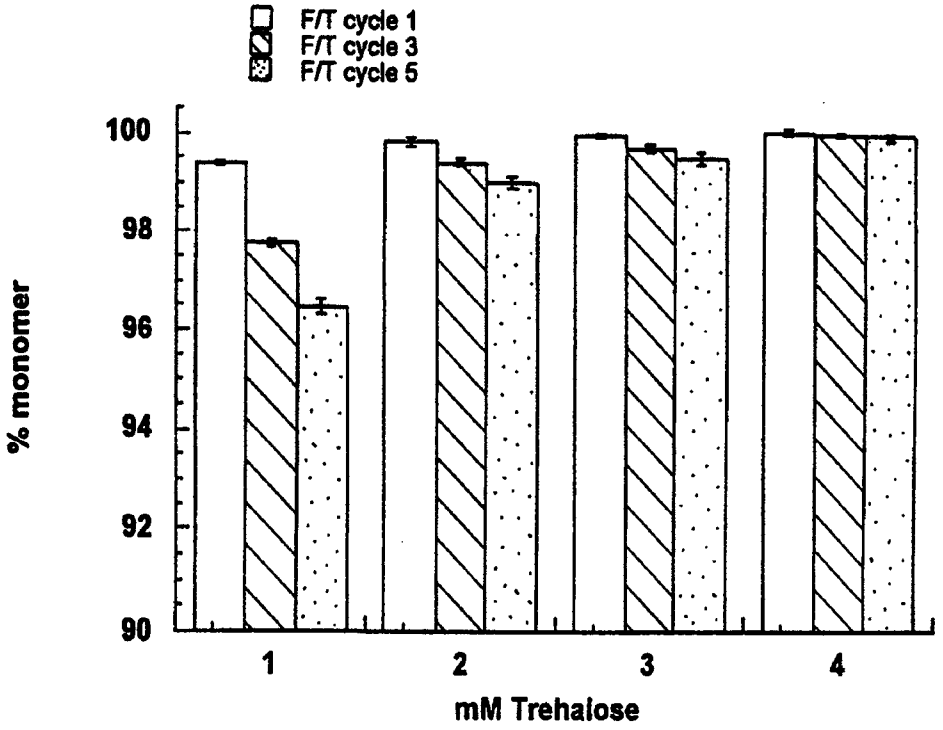

F/T cycle 1
F/T cycle 3
F/T cycle 5
% monomer
100
98
96
94
92
90
1
2
3
4
mM Trehalose

FIG. 25

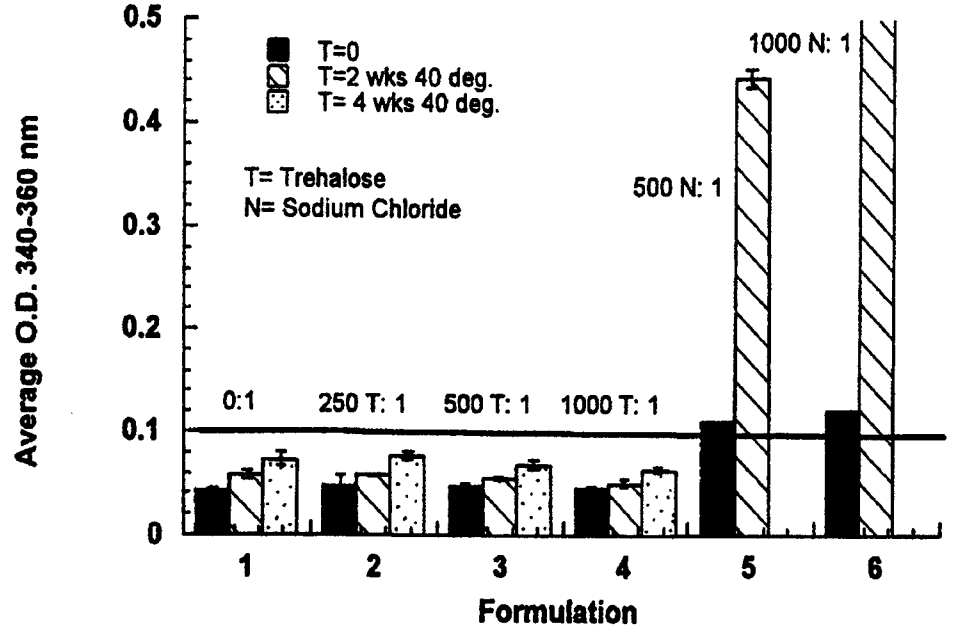

Average O.D. 340-360 nm
0.5
0.4
0.3
0.2
0.1
0
T=0
T=2 wks 40 deg.
T= 4 wks 40 deg.
T= Trehalose
N= Sodium Chloride
1000 N: 1
500 N: 1
0:1
250 T: 1
500 T: 1
1000 T: 1
1
2
3
4
5
6
Formulation

FIG. 26

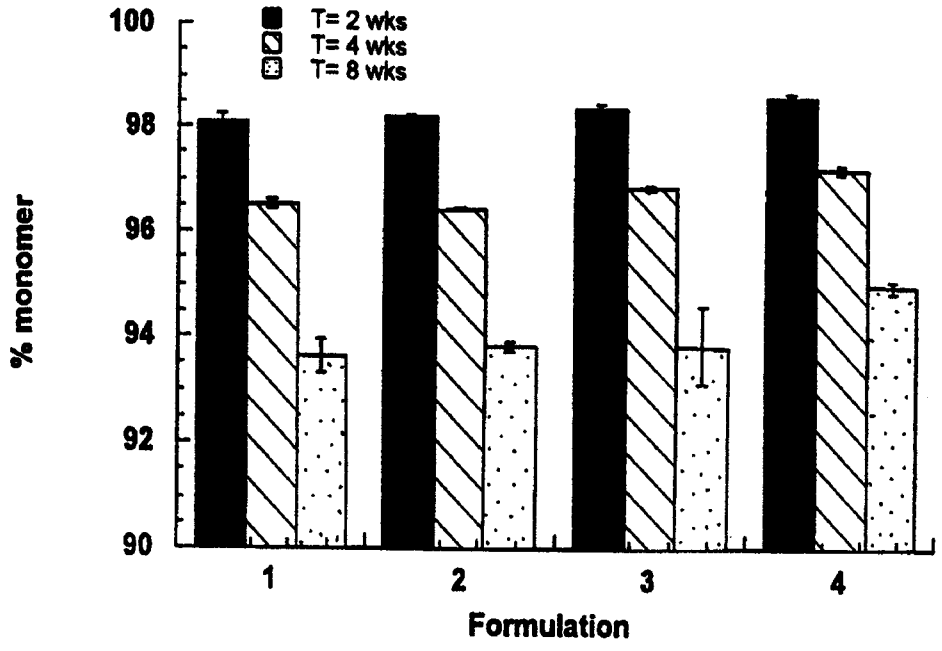

100
98
96
94
92
90
% monomer
T= 2 wks
T= 4 wks
T= 8 wks
1
2
3
4
Formulation

FIG. 27

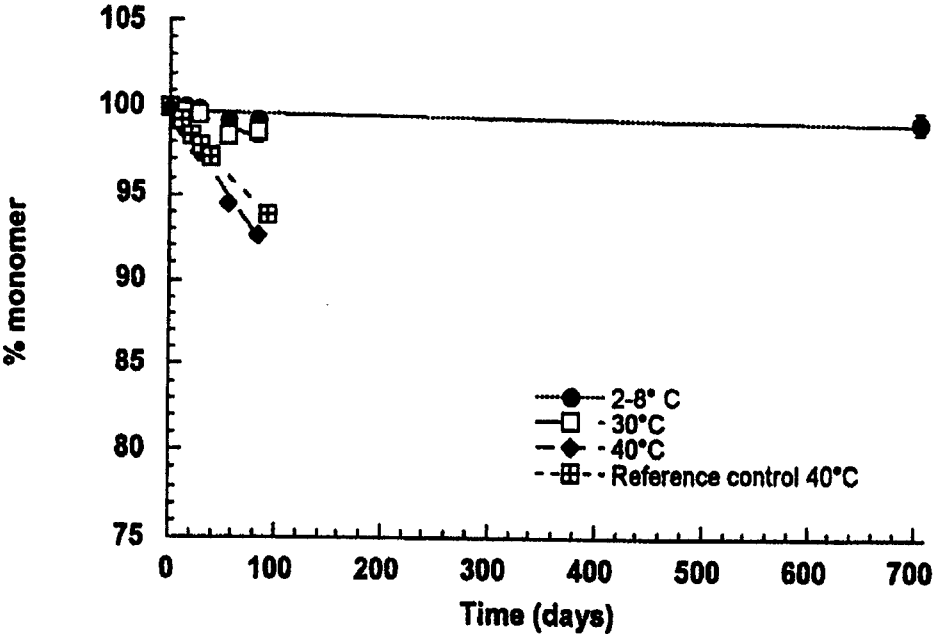

105
100
95
90
85
80
75
% monomer
2-8° C
30°C
40°C
Reference control 40°C
0
100
200
300
400
500
600
700
Time (days)

FIG. 28

US006267958B1

(12) **United States Patent**
Andya et al.

(10) **Patent No.: US 6,267,958 B1**
(45) **Date of Patent: *Jul. 31, 2001**

(54) **PROTEIN FORMULATION**

(75) Inventors: **James Andya**, Millbrae; **Jeffrey L. Cleland**, San Carlos; **Chung C. Hsu**, Los Altos Hills; **Xanthe M. Lam**, San Francisco; **David E. Overcashier**, El Granada; **Steven J. Shire**, Belmont; **Janet Yu-Feng Yang**, San Mateo; **Sylvia Sau-Yan Wu**, San Francisco, all of CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/615,369**

(22) Filed: **Mar. 14, 1996**

Related U.S. Application Data

(60) Provisional application No. 60/029,182, filed on Jul. 27, 1996, now abandoned.

(51) **Int. Cl.**[7] **A61K 39/395**; C07K 16/00

(52) **U.S. Cl.** **424/130.1**; 424/138.1; 424/141.1; 424/155.1; 424/143.1; 424/145.1; 514/2; 530/350; 530/387.1; 530/387.7; 530/388.22; 530/388.8; 530/388.1; 435/810

(58) **Field of Search** 514/2; 424/138.1, 424/130.1, 141.1, 155.1, 143.1, 145.1; 530/350, 387.7, 387.1, 388.1, 388.24, 388.8; 435/810

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,093,606	*	6/1978	Coval .
4,499,073	*	2/1985	Tenold .
4,877,608		10/1989	Lee et al. .
4,891,319	*	1/1990	Rosen .
4,940,782	*	7/1990	Rup .
5,032,405		7/1991	Huang et al. .
5,036,049		7/1991	Audhyu et al. .
5,096,885		3/1992	Pearlman et al. .
5,149,653		9/1992	Roser .
5,215,743		6/1993	Maninder et al. .
5,262,296		11/1993	Ogawa et al. .
5,399,670		3/1995	Bhattacharya et al. .
5,410,025		4/1995	Moller et al. .
5,514,554	*	5/1996	Bacus .
5,571,894	*	11/1996	Wels .
5,580,856	*	12/1996	Prestrelski .
5,587,458	*	12/1996	King .
5,589,167		12/1996	Cleland et al. .
5,608,038		3/1997	Eibl et al. .
5,725,856		3/1998	Hudziak et al. .
5,763,409		6/1998	Bayol et al. .
5,821,337		10/1998	Carter et al. .
5,849,700	*	12/1998	Sorensen et al. .

FOREIGN PATENT DOCUMENTS

30771/89	7/1989	(AU) .
2138853	6/1995	(CA) .
303746	2/1989	(EP) .
391444	10/1990	(EP) .
0 531 539 A1	3/1993	(EP) .
597101	5/1994	(EP) .
661060	7/1995	(EP) .
841067	5/1998	(EP) .
84/00890	3/1984	(WO) .
WO 89/09402	3/1989	(WO) .
WO 89/11297	11/1989	(WO) .
WO 90/11091	4/1990	(WO) .
WO 92/22653	12/1992	(WO) .
WO 93/05799	4/1993	(WO) .
WO 98/22136	5/1998	(WO) .

OTHER PUBLICATIONS

Cleland et al. Proceed. Intern. Symp. Control Rel. Reach. Mater 22:514, 1995.*

Draber et al. J. Imm. Methods 181: 37 1995.*

Bam et al 1995 Pharm. Res. 12:12.*

Pikal et al 1991 Pharm Res 8: 427.*

Baselga et al., "Phase II Study of Weekly Intravenous Recombinant Humanized Anti-p185/HER2 Monoclonal Antibody in Patients With HER2/neu-Overexpressing Metastatic Breast Cancer" *J. Clin. Oncol.* 14(3) :737-744 (1996).

Boulet et al., "Inhibitory Effects of an Anti-IgE Antibody E25 on Allergen-induced Early Asthmatic Response" *Am J Respir Crit Care Med* 155:1835-1840 (1997).

Chang et al., "Development of a Stable Freeze-dried Formulation of Recombinant Human Interleukin-1 Receptor Antagonist" *Pharmaceutical Research* 13(2) :243-248 (1996).

Fahy et al., "The effect of an Anti-IgE monoclonal natibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects" *Am J Respir Crit Care Med* 155:1828-1834 (1997).

(List continued on next page.)

Primary Examiner—Christina Y. Chan
Assistant Examiner—Marianne DiBrino
(74) *Attorney, Agent, or Firm*—Wendy M. Lee

(57) **ABSTRACT**

A stable lyophilized protein formulation is described which can be reconstituted with a suitable diluent to generate a high protein concentration reconstituted formulation which is suitable for subcutaneous administration. For example, anti-IgE and anti-HER2 antibody formulations have been prepared by lyophilizing these antibodies in the presence of a lyoprotectant. The lyophilized mixture thus formed is reconstituted to a high protein concentration without apparent loss of stability of the protein.

47 Claims, 10 Drawing Sheets

OTHER PUBLICATIONS

"Immune Globulin Intravenous (Human) Sandoglobulin" *Product Information, Physicians' Desk Reference* pp. 2075–2077 (1994).

"IVEEGAM", "Product Information, IMMUNO U.S., Inc." pp. 2 pages (1992).

"Sandoglobulin" *Physicians' Desk Reference* pp. 1965–1966 (1991).

Arakawa et al., "Protein–Solvent Interactions in Pharmaceutical Formulations" *Pharmaceutical Research* 8(3):285–291 (1991).

Beauvais et al., "Both Glassy State and Native Structure are Required for Storage Stability of Lyophilized Interleukin–1 Receptor Antagonist" *Pharm. Res.* (Abstract #2007) 12(9):S–80 (1995).

Chang and Fischer, "Development of an Efficient Single–Step Freeze–Drying Cycle for Protein Formulations" *Pharm. Res.* 12 (6) :831–837 (1995).

Cleland and Jones, "Development of Stable Protein Formulations for Microencapsulation in Biodegradable Polymers" *Proceed. Intern. Symp. Control. Rel. Bioact. Mater.* 22:514–515 (1995).

Cleland et al., "The Development of Stable Protein Formulations: A Close Look at Protein Aggregation, Deamidation, and Oxidation" *Critical Reviews in Therapeutic Drug Carrier Systems* 10(4) :307–377 (1993).

Draber et al., "Stability of Monoclonal IgM Antibodies Freeze–Dried in the Presence of Trehalose" *Journal of Immunological Methods* 181(1) :37–43 (1995).

Izutsu et al., "The effects of additives on the stability of freeze–dried B–galactosidase storeed at elevated etmperature" *Intl. J. Pharmaceutics* 71:137–146 (1991).

Manning et al., "Stability of Protein Pharmaceuticals" *Pharm. Res.* 6(11) :903–918 (1989).

Nielson, et al., "Stability of Freeze Dried Horseradish Peroxidase Conjugated Monclonal Antibodies Used in Diagnostic Serology" *Journal of Immunoassay* 16(2) :183–187 (1995).

Pearlman et al., "Analysis of Protein Drugs" *Peptide and Protein Drug Delivery*, Vincent H. L. Lee, Marcel Dekker, Inc. pp. 247–301 (1991).

Pikal, M., "Freeze–Drying of Proteins, Part 2: Formulation Selection" *Biopharm.* 3(9) :26–30 (1990).

Presta et al., "Humanization of an Antibody Directed Against IgE" *J. Immunol.* 151(5) :2623–2632 Sep. 1, 1993.

Wang et al., "Parenteral Formulations of Proteins and Peptides: Stability and Stabilizers" *J. Parental Sci. Tech.* (Technical Report No. 10) 42(2S) :S4–S26 (1988).

* cited by examiner

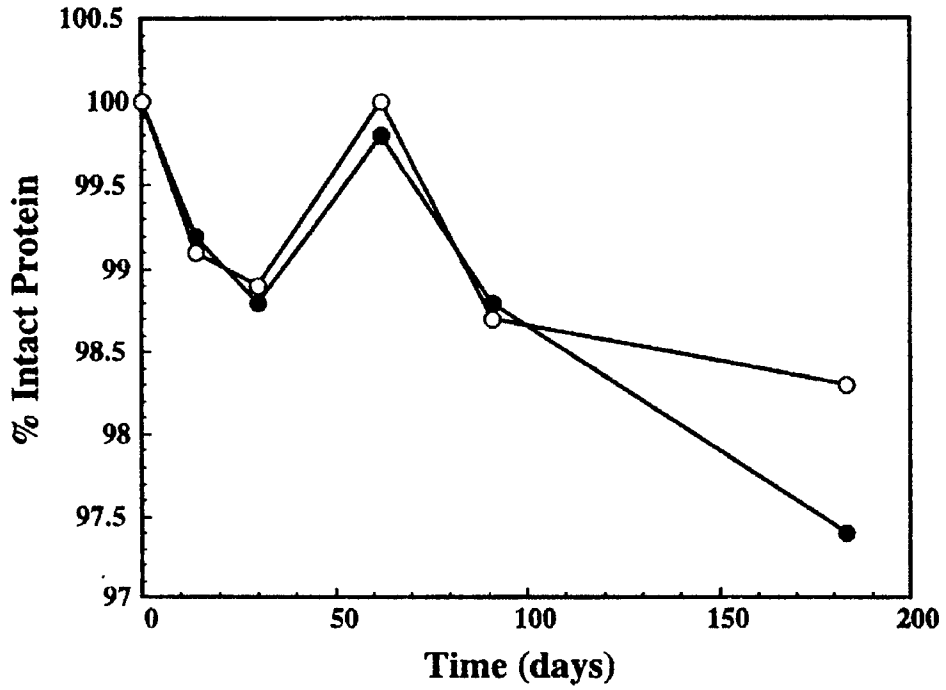

100.5
100
99.5
99
98.5
98
97.5
97
% Intact Protein
0
50
100
150
200
Time (days)

FIG. 1

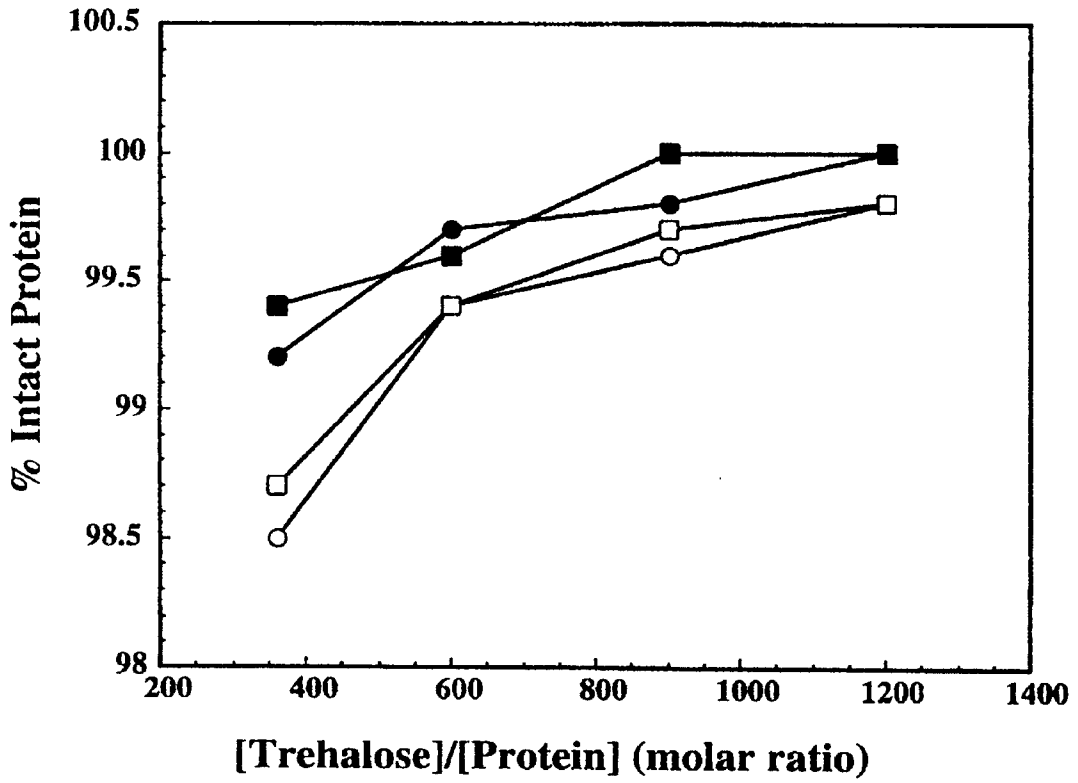

100.5
100
99.5
99
98.5
98
% Intact Protein
200
400
600
800
1000
1200
1400
[Trehalose]/[Protein] (molar ratio)

FIG. 2

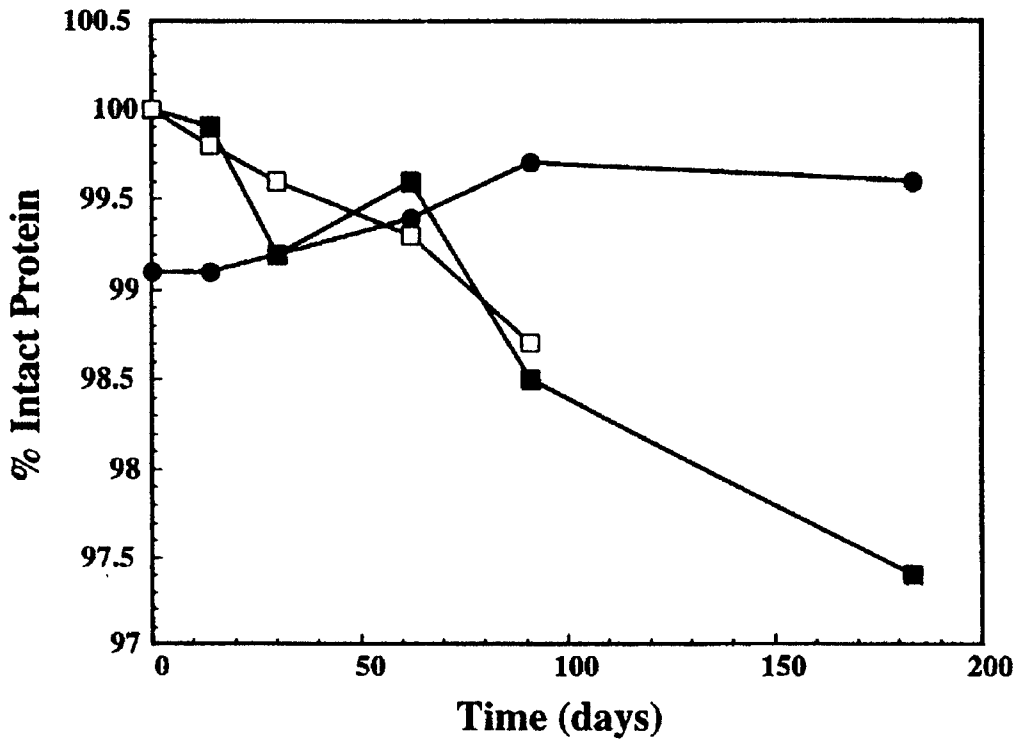

100.5
100
99.5
99
98.5
98
97.5
97
% Intact Protein
0
50
100
150
200
Time (days)

FIG. 3

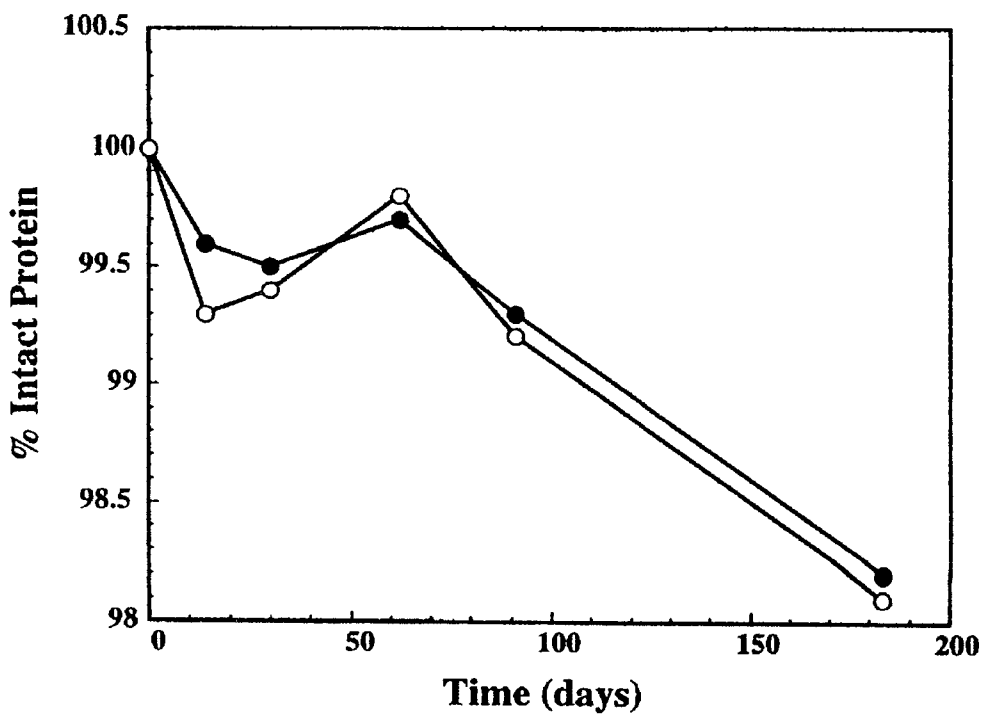

100.5
100
99.5
99
98.5
98
% Intact Protein
0
50
100
150
200
Time (days)

FIG. 4

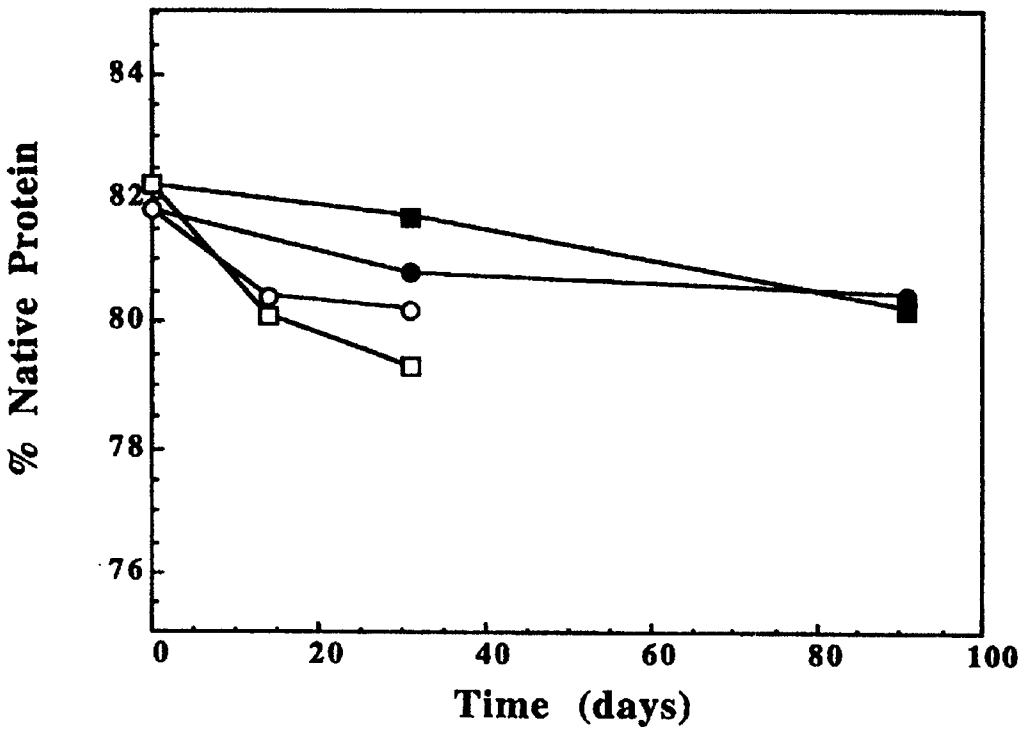

% Native Protein
84
82
80
78
76
0
20
40
60
80
100
Time (days)

FIG. 5

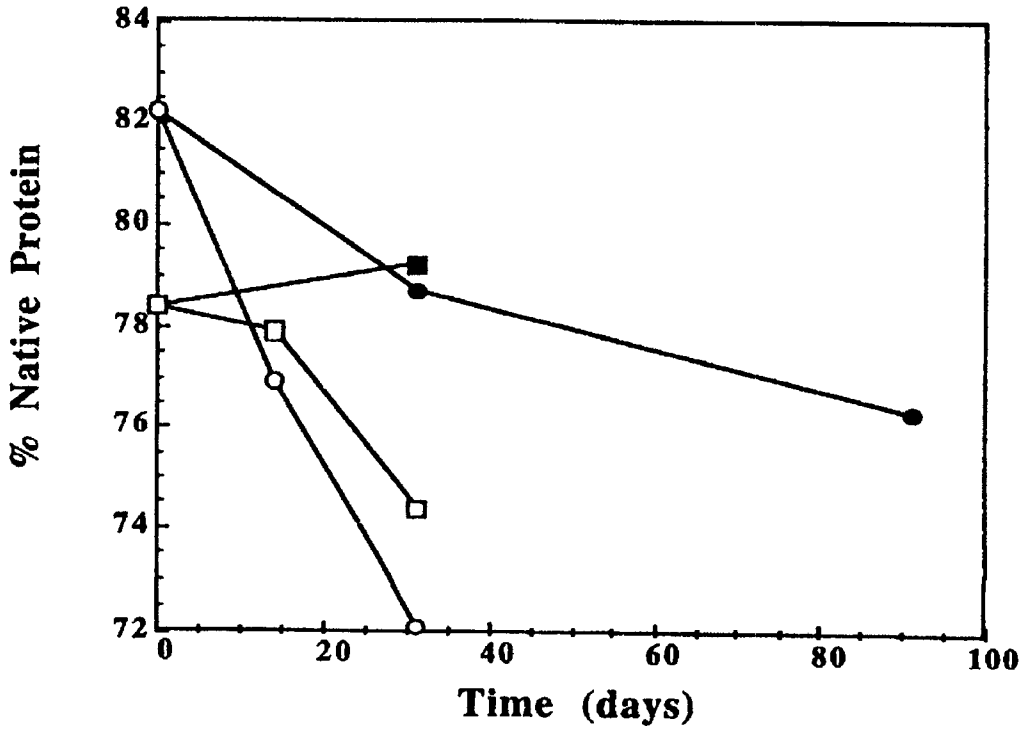

% Native Protein
84
82
80
78
76
74
72
0
20
40
60
80
100
Time (days)

FIG. 6

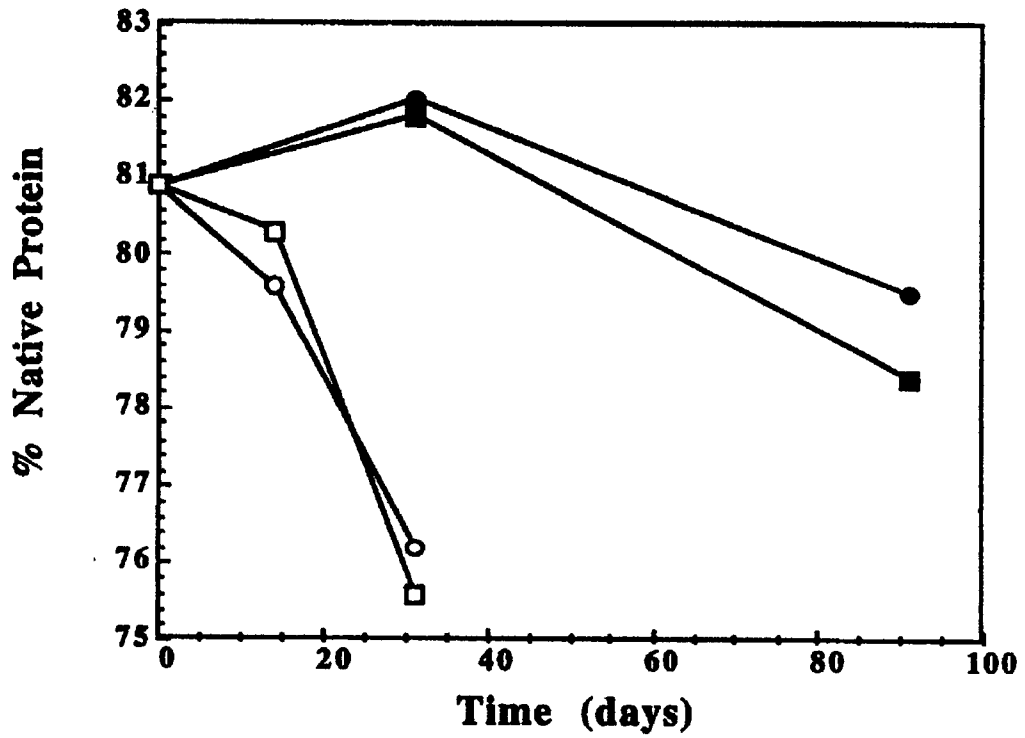

% Native Protein
83
82
81
80
79
78
77
76
75
0
20
40
60
80
100
Time (days)

FIG. 7

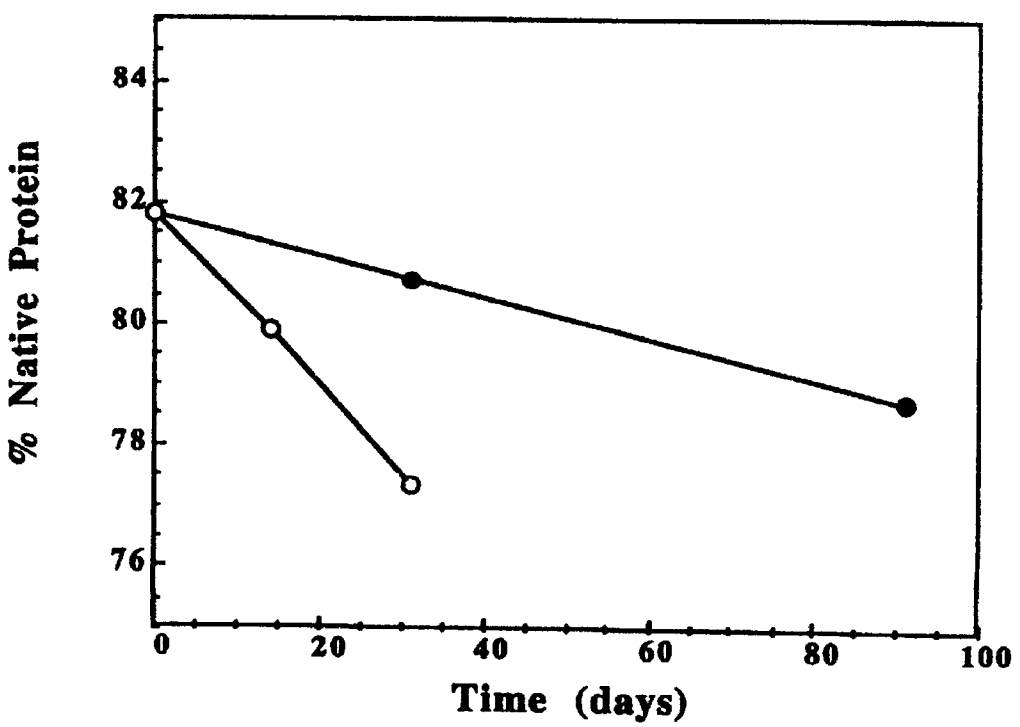

% Native Protein
84
82
80
78
76
0
20
40
60
80
100
Time (days)

FIG. 8

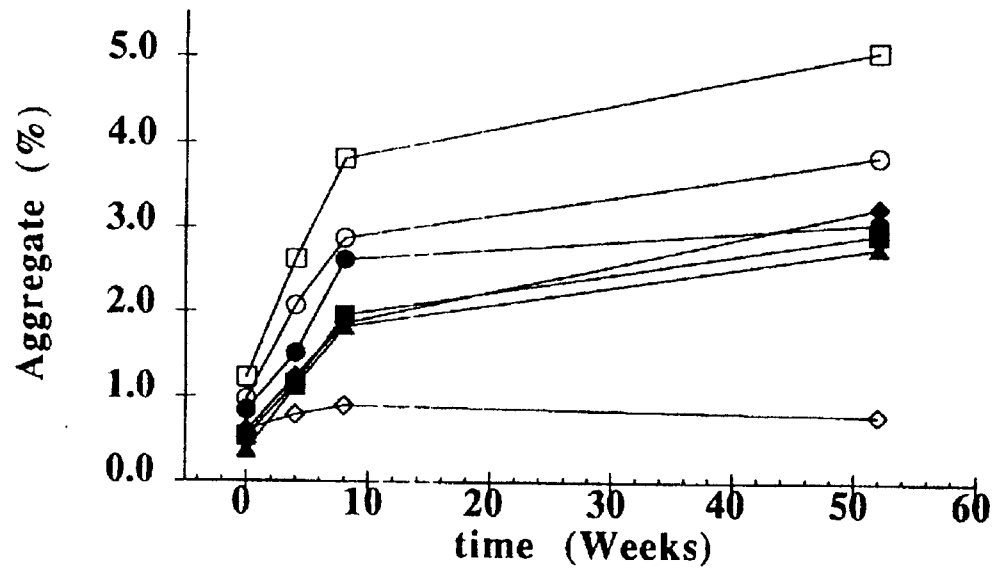

Aggregate (%)
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
50
60
time (Weeks)

FIG. 9

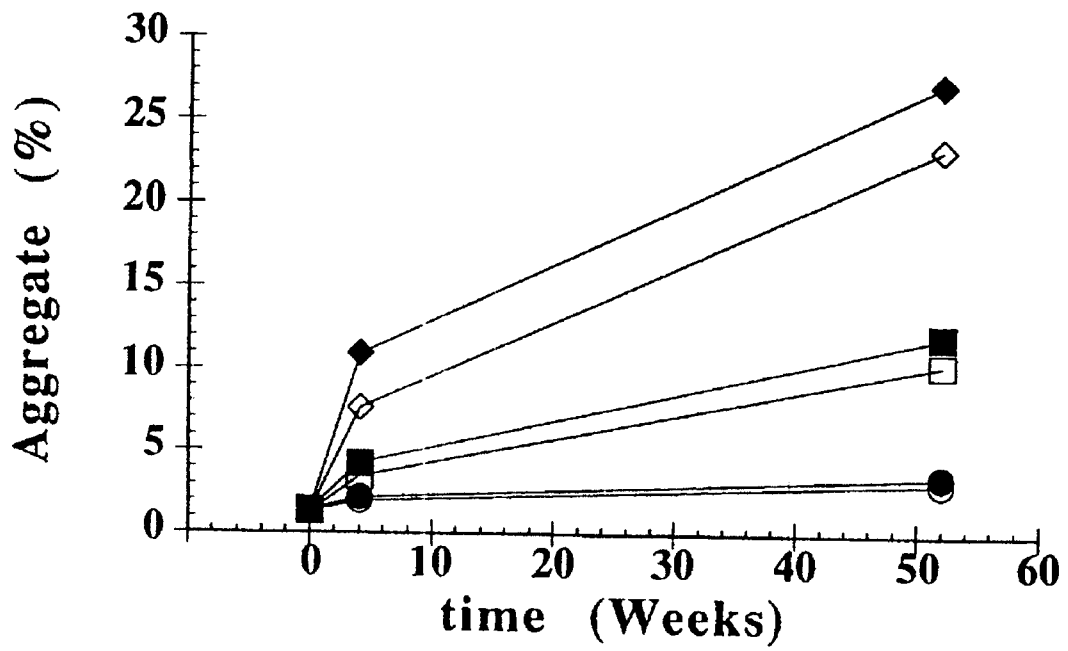

Aggregate (%)
30
25
20
15
10
5
0
0
10
20
30
40
50
60
time (Weeks)

FIG. 10

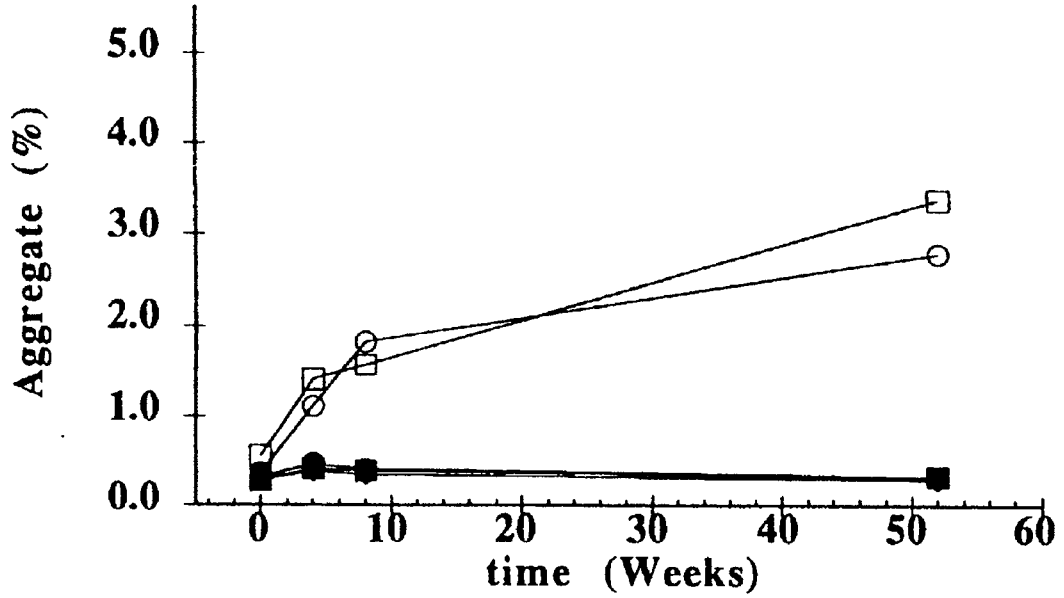

5.0
4.0
3.0
2.0
1.0
0.0
Aggregate (%)
0
10
20
30
40
50
60
time (Weeks)

FIG. 11

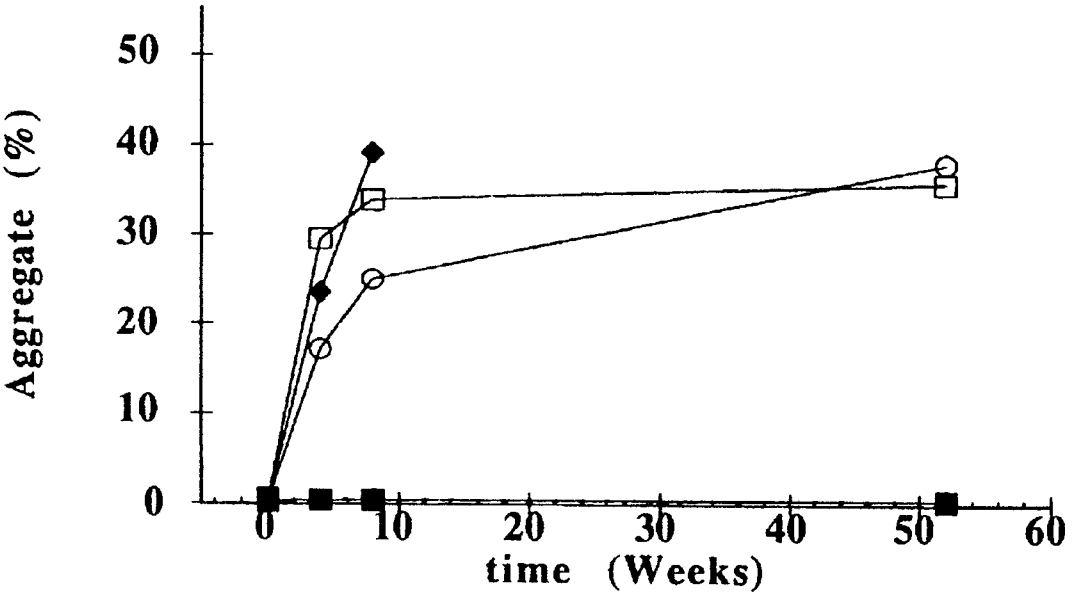

50
40
30
20
10
0
Aggregate (%)
0
10
20
30
40
50
60
time (Weeks)

FIG. 12

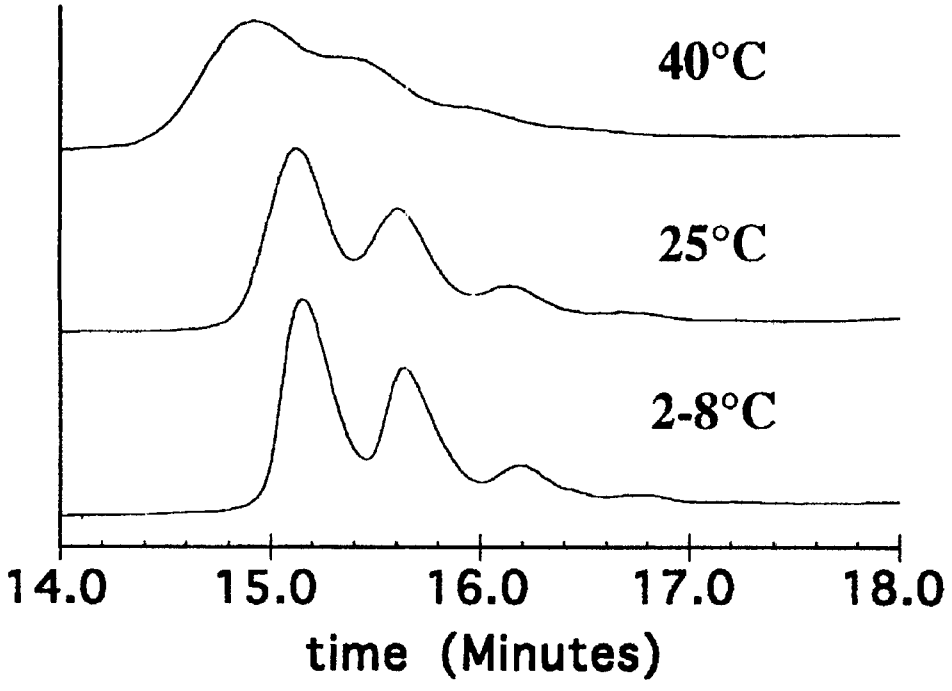

40°C
25°C
2-8°C
14.0
15.0
16.0
17.0
18.0
time (Minutes)

FIG. 13

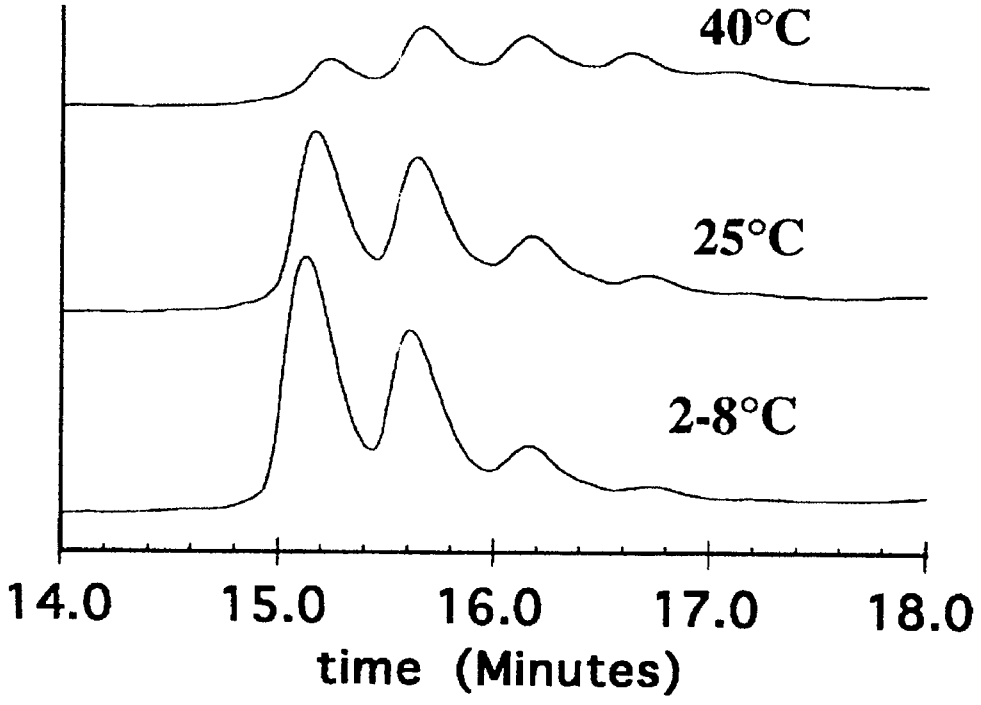

40°C
25°C
2-8°C
14.0
15.0
16.0
17.0
18.0
time (Minutes)

FIG. 14

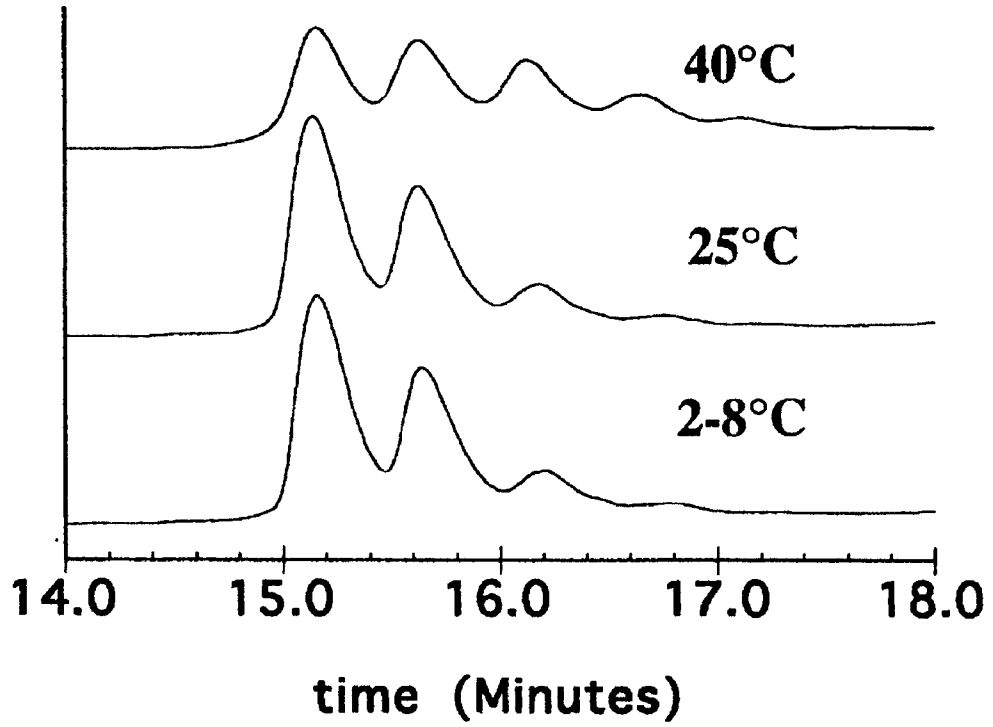

40°C
25°C
2-8°C
14.0
15.0
16.0
17.0
18.0
time (Minutes)

FIG. 15

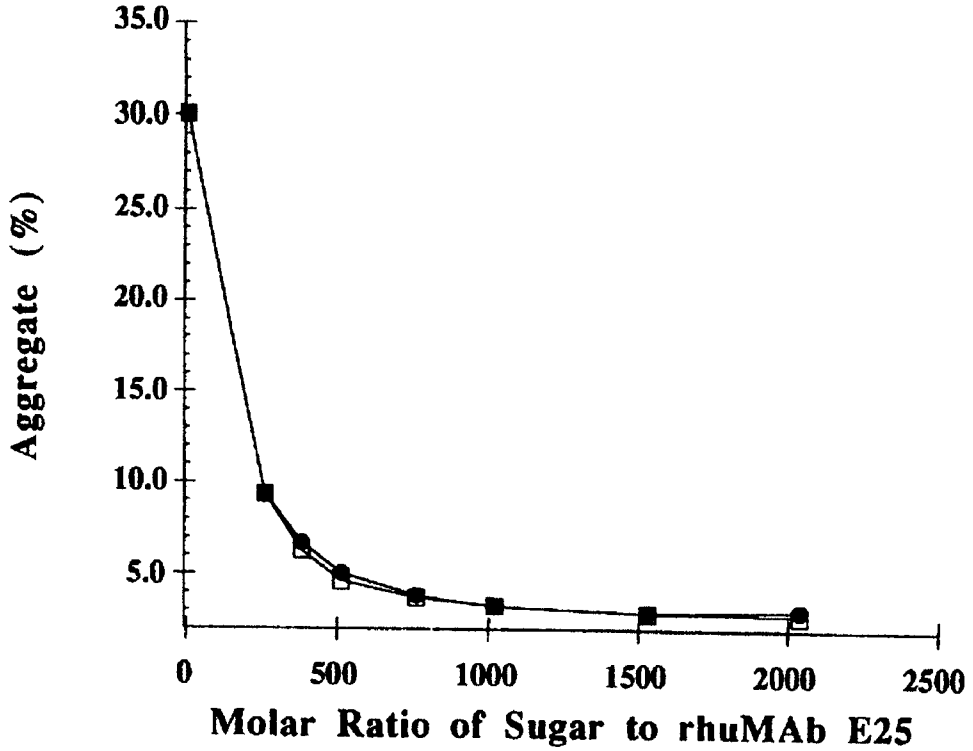

35.0
30.0
25.0
20.0
15.0
10.0
5.0
Aggregate (%)
0
500
1000
1500
2000
2500
Molar Ratio of Sugar to rhuMAb E25

FIG. 16

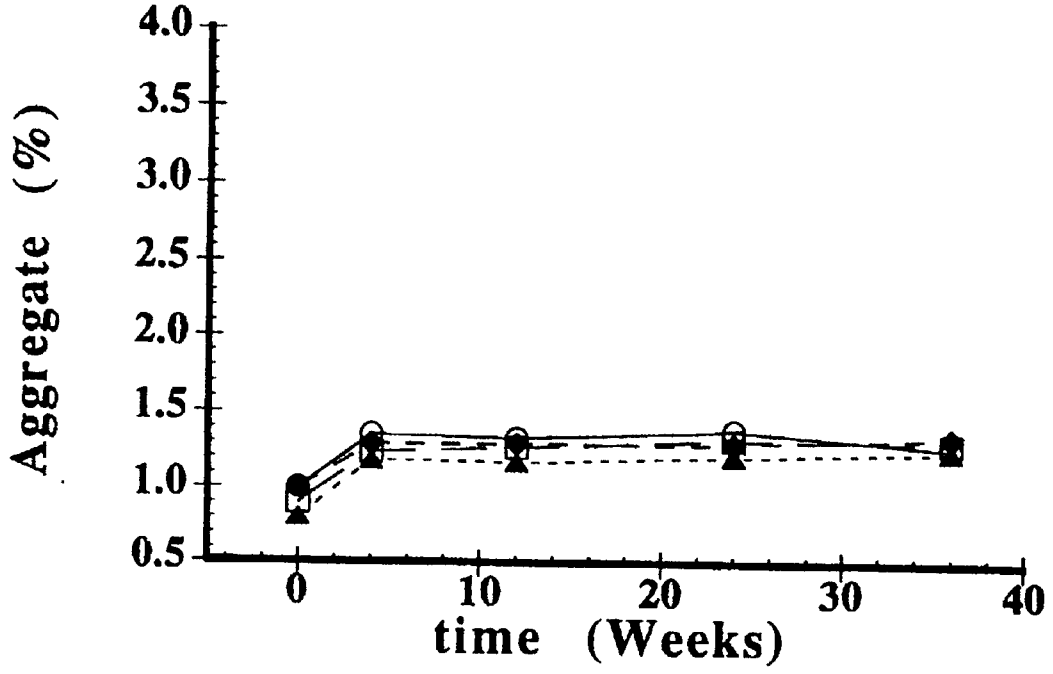

Aggregate (%)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
10
20
30
40
time (Weeks)

FIG. 17

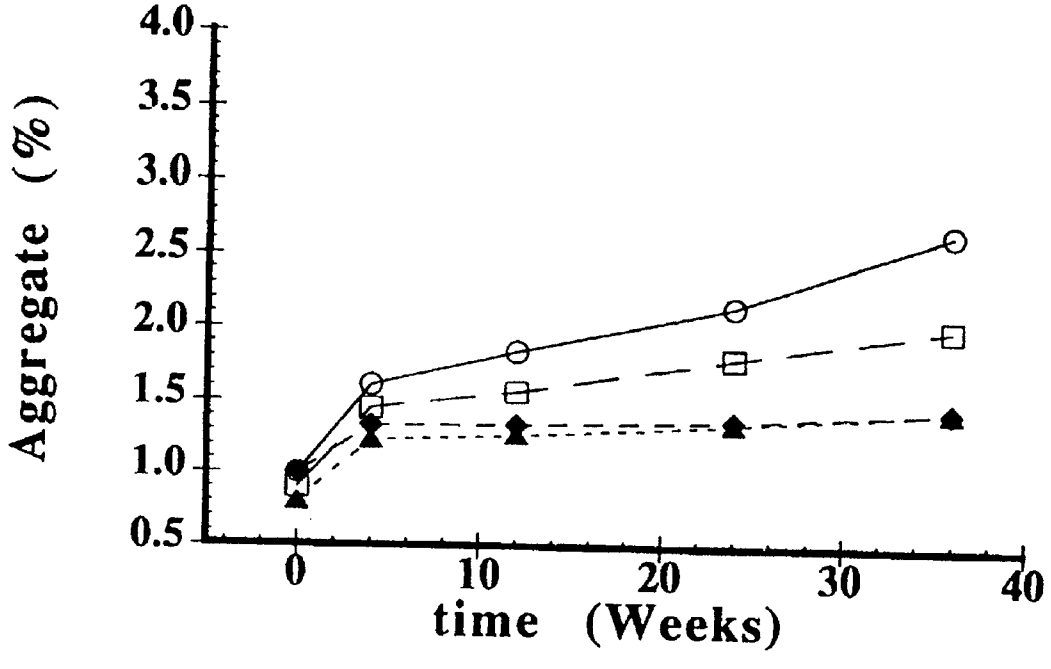

Aggregate (%)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
10
20
30
40
time (Weeks)

FIG. 18

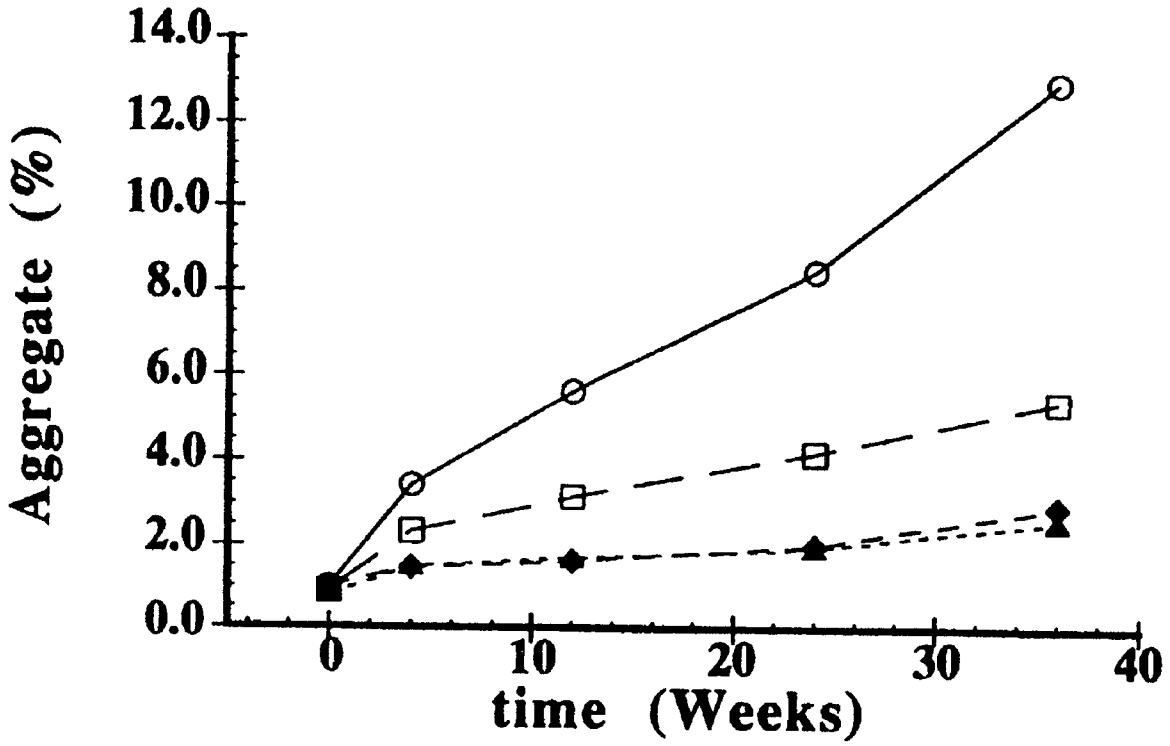


FIG. 19

PROTEIN FORMULATION

This application is a non-provisional application filed under 37 CFR 1.53(b) claiming priority under 35 USC 119(e) to provisional application 60/029,182 converted from non-provisional application Ser. No. 08/508,014 filed Jul. 27, 1996 now abandoned, the contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention is directed to a lyophilized protein formulation. In particular, it relates to a stable lyophilized protein formulation which can be reconstituted with a diluent to generate a stable reconstituted formulation suitable for subcutaneous administration.

2. Description of Related Disclosures

In the past ten years, advances in biotechnology have made it possible to produce a variety of proteins for pharmaceutical applications using recombinant DNA techniques. Because proteins are larger and more complex than traditional organic and inorganic drugs (i.e. possessing multiple functional groups in addition to complex three-dimensional structures), the formulation of such proteins poses special problems. For a protein to remain biologically active, a formulation must preserve intact the conformational integrity of at least a core sequence of the protein's amino acids while at the same time protecting the protein's multiple functional groups from degradation. Degradation pathways for proteins can involve chemical instability (i.e. any process which involves modification of the protein by bond formation or cleavage resulting in a new chemical entity) or physical instability (i.e. changes in the higher order structure of the protein). Chemical instability can result from deamidation, racemization, hydrolysis, oxidation, beta elimination or disulfide exchange. Physical instability can result from denaturation, aggregation, precipitation or adsorption, for example. The three most common protein degradation pathways are protein aggregation, deamidation and oxidation. Cleland et al. *Critical Reviews in Therapeutic Drug Carrier Systems* 10(4): 307–377 (1993).

Freeze-drying is a commonly employed technique for preserving proteins which serves to remove water from the protein preparation of interest. Freeze-drying, or lyophilization, is a process by which the material to be dried is first frozen and then the ice or frozen solvent is removed by sublimation in a vacuum environment. An excipient may be included in pre-lyophilized formulations to enhance stability during the freeze-drying process and/or to improve stability of the lyophilized product upon storage. Pikal, M. *Biopharm.* 3(9)26–30 (1990) and Arakawa et al. *Pharm. Res.* 8(3):285–291 (1991).

It is an object of the present invention to provide a lyophilized protein formulation which is stable upon storage and delivery. It is a further object to provide a stable reconstituted protein formulation which is suitable for subcutaneous administration. In certain embodiments, it is an object to provide a multi-use formulation which is stable for at least the time over which it will be administered to a patient.

SUMMARY OF THE INVENTION

This invention is based on the discovery that a stable lyophilized protein formulation can be prepared using a lyoprotectant (preferably a sugar such as sucrose or trehalose), which lyophilized formulation can be reconstituted to generate a stable reconstituted formulation having a protein concentration which is significantly higher (e.g. from about 2–40 times higher, preferably 3–10 times higher and most preferably 3–6 times higher) than the protein concentration in the pre-lyophilized formulation. In particular, while the protein concentration in the pre-lyophilized formulation may be 5 mg/mL or less, the protein concentration in the reconstituted formulation is generally 50 mg/mL or more. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where the formulation is intended for subcutaneous administration. Despite the very high protein concentration in the reconstituted formulation, it has been found that the reconstituted formulation is stable (i.e. fails to display significant or unacceptable levels of chemical or physical instability of the protein) at 2–8° C. for at least about 30 days. In certain embodiments, the reconstituted formulation is isotonic. In spite of the use of lower concentrations of the lyoprotectant to achieve such isotonic formulations upon reconstitution, it was discovered herein that the protein in the lyophilized formulation essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

When reconstituted with a diluent comprising a preservative (such as bacteriostatic water for injection, BWFI), the reconstituted formulation may be used as a multi-use formulation. Such a formulation is useful, for example, where the patient requires frequent subcutaneous administrations of the protein to treat a chronic medical condition. The advantage of a multi-use formulation is that it facilitates ease of use for the patient, reduces waste by allowing complete use of vial contents, and results in a significant cost savings for the manufacturer since several doses are packaged in a single vial (lower filling and shipping costs).

Based on the observations described herein, in one aspect the invention provides a stable isotonic reconstituted formulation comprising a protein in an amount of at least about 50 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of a protein and a lyoprotectant, wherein the protein concentration in the reconstituted formulation is about 2–40 times greater than the protein concentration in the mixture before lyophilization.

In another embodiment, the invention provides a stable reconstituted formulation comprising an antibody in an amount of at least about 50 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of an antibody and a lyoprotectant, wherein the antibody concentration in the reconstituted formulation is about 2–40 times greater than the antibody concentration in the mixture before lyophilization.

The ratio of lyoprotectant:protein in the lyophilized formulation of the preceding paragraphs depends, for example, on both the protein and lyoprotectant of choice, as well as the desired protein concentration and isotonicity of the reconstituted formulation. In the case of a full length antibody (as the protein) and trehalose or sucrose (as the lyoprotectant) for generating a high protein concentration isotonic reconstituted formulation, the ratio may, for example, be about 100–1500 mole trehalose or sucrose: 1 mole antibody.

Generally, the pre-lyophilized formulation of the protein and lyoprotectant will further include a buffer which provides the formulation at a suitable pH, depending on the protein in the formulation. For this purpose, it has been found to be desirable to use a histidine buffer in that, as demonstrated below, this appears to have lyoprotective properties.

The formulation may further include a surfactant (e.g. a polysorbate) in that it has been observed herein that this can reduce aggregation of the reconstituted protein and/or reduce the formation of particulates in the reconstituted formulation. The surfactant can be added to the pre-

lyophilized formulation, the lyophilieed formulation and/or the reconstituted formulation (but preferably the pre-lyophilized formulation) as desired.

The invention further provides a method for preparing a stable isotonic reconstituted formulation comprising reconstituting a lyophilized mixture of a protein and a lyoprotectant in a diluent such that the protein concentration in the reconstituted formulation is at least 50 mg/mL, wherein the protein concentration in the reconstituted formulation is about 2–40 times greater than the protein concentration in the mixture before lyophilization.

In yet a further embodiment, the invention provides a method for preparing a formulation comprising the steps of: (a) lyophilizing a mixture of a protein and a lyoprotectant; and (b) reconstituting the lyophilized mixture of step (a) in a diluent such that the reconstituted formulation is isotonic and stable and has a protein concentration of at least about 50 mg/mL. For example, the protein concentration in the reconstituted formulation maybe from about 80 mg/hn to about 300 mg/mL. Generally, the protein concentration in the reconstituted formulation is about 2–40 times greater than the protein concentration in the mixture before lyophilization.

An article of manufacture is also provided herein which comprises: (a) a container which holds a lyophilized mixture of a protein and a lyoprotectant; and (b) instructions for reconstituting the lyophilized mixture with a diluent to a protein concentration in the reconstituted formulation of at least about 50 mg/mL. The article of manufacture may further comprise a second container which holds a diluent (e.g. bacteriostatic water for injection (BWFI) comprising an aromatic alcohol).

The invention further provides a method for treating a mammal comprising administering a therapeutically effective amount of a reconstituted formulation disclosed herein to a manmmal, wherein the mammal has a disorder requiring treatment with the protein in the formulation. For example, the formulation may be administered subcutaneously.

One useful anti-HER2 antibody pre-lyophilized formulation as discovered in the experiments detailed below was found to comprise anti-HER2 in amount from about 5–40 mg/mL (e.g 20–30 mg/mL) and sucrose or trehalose in an amount from about 10–100 mM (e.g 40–80 mM), a buffer (e.g histidine, pH 6 or succinate, pH 5) and a surfactant (e.g. a polysorbate). The lyophilized formulation was found to be stable at 40 C for at least 3 months and stable at 30° C. for at least 6 months. This anti-HER2 formulation can be reconstituted with a diluent to generate a formulation suitable for intravenous administration comprising anti-HER2 in an amount from about 10–30 mg/mL which is stable at 2–80 C for at least about 30 days. Where higher concentrations of the anti-HER2 antibody are desired (for example where subcutaneous delivery of the antibody is the intended mode of administration to the patient), the lyophilized formulation may be reconstituted to yield a stable reconstituted formulation having a protein concentration of 50 mg/mL or more.

One desirable anti-IgE antibody pre-lyophilized formulation discovered herein has anti-IgE in amount from about 5–40 mg/nl (e.g. 20–30 mg/nl) and sucrose or trehalose in an amount from about 60– 300 mM (e.g. 80–170 mM), a buffer (preferably histidine, pH 6) and a surfactant (such as a polysorbate). The lyophilized anti-IgE formulation is stable at 300C for at least 1 year. This formulation can be reconstituted to yield a formulation comprising anti-IgE in an amount from about 15–45 mg/mL (e.g 15–25 mg/mL) suitable for intravenous administration which is stable at 2–8° C. for at least 1 year. Alternatively, where higher concentrations of anti-IgE in the formulation are desired, the lyophilized formulation can be reconstituted in order to generate a stable formulation having an anti-IgE concentration of >50 mg/mL.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the effect of reconstitution volume on the stability of lyophilized rhuMAb HER2. The lyophilized formulation was prepared from a pre-lyophilization formulation comprising 25 mg/mL protein,60 mM trehalose, 5 mM sodium succinate, pH 5.0, and 0.01% Tween 20™. The lyophilized cake was incubated at 40° C. and then reconstituted with 4.0(○) or 20.0 mL (●) of BWFI. The fraction of intact protein in the reconstituted formulation was measured by native size exclusion chromatography and defined as the peak area of the native protein relative to the total peak area including aggregates.

FIG. **2** illustrates the effect of trehalose concentration on the stability of lyophilized rhuMAb HER2. The protein was lyophilized at 25 mg/mL in 5 mM sodium succinate, pH 5.0 (circles) or 5 mM histidine, pH 6.0 (squares) and trehalose concentrations ranging from 60 mM (360 molar ratio) to 200 mM (1200 molar ratio). The lyophilized protein was incubated at 40° C. for either 30 days (closed symbols) or 91 days (open symbols). The amount of intact protein was measured after reconstitution of the lyophilized protein with 20 mL BWFI.

FIG. **3** demonstrates the effect of trehalose concentration on the long term stability of lyophilized rhuMAb HER2 stored at 40° C. The protein was lyophilized at either 25 mg/mL in 5 mM sodium succinate, pH 5.0, 0.01% Tween 20™, and 60 mM trehalose (■) or 5 mM histidine, pH 6.0, 0.01% Tween 20™, and 60 mM trehalose (□) or 21 mg/mL in 10 mM sodium succinate, pH 5.0, 0.2% Tween 20™ and 250 mM trehalose (●). The lyophilized protein was incubated at 40° C. and then reconstituted with 20 mL of BWFI. The amount of intact protein was measured after reconstitution.

FIG. **4** shows the stability of rhuMAb HER2 lyophilized in 38.4 mM mannitol (7 mg/mL), 20.4 mM sucrose (7 mg/mL), 5 mM histidine, pH 6.0, 0.01% Tween 20™. The lyophilized protein was incubated at 40° C. and then reconstituted with either 4.0 mL (○) or 20 mL (●) of BWFI. The amount of intact protein was measured after reconstitution.

FIG. **5** demonstrates stability of reconstituted rhuMAb HER2 lyophilized in 5 mM sodium succinate, pH 5.0, 60 mM trehalose, 0.01% Tween 20™. Samples were reconstituted with either 4.0 mL (squares) or 20.0 mL (circles) of BWFI (20 mL:0.9% benzyl alcohol; 4 mL: 1.1% benzyl alcohol) and then stored at 5° C. (solid symbols) or 25° C. (open symbols). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

FIG. **6** shows stability of reconstituted rhuMAb HER2 lyophilized in 5 mM histidine, pH 6.0, 60 mM trehalose, 0.01% Tween 20. Samples were reconstituted with either 4.0 mL (squares) or 20.0 mL (circles) of BWFI (20 mL:0.9% benzyl alcohol; 4 mL: 1.1% benzyl alcohol) and then stored at 5° C. (solid symbols) or 25° C. (open symbols). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

FIG. **7** reveals stability of reconstituted rhuMAb HER2 lyophilized in 5 mM histidine, pH 6.0, 38.4 mM mannitol, 20.4 mM sucrose, 0.01% Tween 20. Samples were reconstituted with either 4.0 mL (squares) or 20.0 mL (circles) of BWFI (20 mL:0.9% benzyl alcohol; 4 mL:1.1%benzyl alcohol) andthen stored at 5° C. (solid symbols) or 25° C. (open symbols). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

FIG. **8** shows stability of reconstituted rhuMAb HER2 lyophilized in 10 mM sodium succinate, pH 5.0, 250 mM trehalose, 0.2% Tween 20. Samples were reconstituted with 20.0 mL of BWFI (0.9% benzyl alcohol) and then stored at 5° C. (●) or 25° C. (○). The % native protein was defined as the peak area of the native (not degraded) protein relative to the total peak area as measured by cation exchange chromatography.

FIG. **9** shows aggregation of rhuMAb E25 formulated into buffers ranging from pH 5 to pH 7 at 10 mM buffer concentration and 5 mg/mL antibody concentration. Samples were lyophilized and assayed at time zero and after 4 weeks, 8 weeks, and 52 weeks of storage at 2–8° C. The buffers were: potassium phosphate pH 7.0 (○); sodium phosphate pH 7.0 (□); histidine pH 7.0 (◇); sodium succinate pH 6.5 (●); sodium succinate pH 6.0 (■); sodium succinate pH 5.5 (♦); and sodium succinate pH 5.0 (▲).

FIG. **10** depicts aggregation of rhuMAb E25 lyophilized in 5 mM histidine buffer at both pH 6 and pH 7 and assayed following storage as follows. The buffer was at: pH 6.0 stored at 2–8° C. (○); pH 6 stored at 25° C. (□); pH 6 stored at 40° C. (◇); pH 7 stored at 2–8° C. (●); pH 7 stored at 25° C. (■); and pH 7 stored at 40° C. (♦).

FIG. **11** illustrates aggregation of 5 mg/nL rhuMAb E25 formulated into 10 mM sodium succinate at pH 5.0 with lyoprotectant added at a concentration of 275 mM (isotonic). The lyoprotectants were: control, no lyoprotectant (○); mannitol (□); lactose (◇); maltose (●); trehalose (■); and sucrose (♦). Samples were lyophilized and assayed at time zero and after 4 weeks, 8 weeks, and 52 weeks of storage at 2–8° C.

FIG. **12** shows aggregation of 5 mg/mL rhuMAb E25 formulated into 10 mM sodium succinate at pH 5.0 with lyoprotectant added at a concentration of 275 mM (isotonic). The lyoprotectants were: control, no lyoprotectant (○); mannitol (□); lactose (◇); maltose (●); trehalose (■); and sucrose (♦). Samples were lyophilized and assayed at time zero and after 4 weeks, 8 weeks, and 52 weeks of storage at 40° C.

FIG. **13** depicts hydrophobic interaction chromatography of 20 mg/mL rhuMAb E25 lyophilized in histidine buffer at pH 6 with an isotonic concentration (i.e. 275 mM) of lactose stored for 24 weeks at 2–8, 25 or 40° C. and reconstituted to 20 mg/mL.

FIG. **14** shows hydrophobic interaction chromatography of 20 mg/mL rhuMAb E25 lyophilized in histidine buffer at pH 6 stored for 24 weeks at 2–8, 25 or 40° C. and reconstituted to 20 mg/mL.

FIG. **15** illustrates hydrophobic interaction chromatography of 20 mg/mL rhuMAb E25 lyophilized in histidine buffer at pH 6 with an isotonic concentration (i. e. 275 mM) of sucrose and stored for 24 weeks at 2–8, 25 or 40° C. and reconstituted to 20 mg/mL.

FIG. **16** illustrates the effect of sugar concentration on rhuMAb E25 formulated at 20 mg/mL in 5 mM histidine at pH 6.0. Sucrose (●) and trehalose (□) were added to the formulation at molar ratios ranging from 0 to 2010 (isotonic) (see Table 1 below). Samples were lyophilized and assayed after 12 weeks of storage at 50° C.

TABLE 1

Moles of Sugar: E25 antibody	Sugar conc. (mM)
0	0
260	34.4
380	51.6

TABLE 1-continued

Moles of Sugar: E25 antibody	Sugar conc. (mM)
510	61.8
760	103.1
1020	137.5
1530	206.3
2010	275

FIG. **17** reveals aggregation of rhuMAb E25 formulated at 25 mg/mL into 5 mM histidine at pH 6 with 85 mM sucrose (○); 85 mM trehalose (□); 161 mM sucrose (♦) or 161 mM trehalose (▲). Samples were lyophilized and stored at 2–8° C. followed by reconstitution with 0.9% benzyl alcohol to 100 mg/mL antibody in 20 mM histidine at pH 6 with isotonic (340 mM) and hypertonic (644 mM) sugar concentration.

FIG. **18** shows aggregation of rhuMAb E25 formulated at 25 mg/mL into 5 mM histidine at pH 6 with 85 mM sucrose (○); 85 mM trehalose (□); 161 mM sucrose (♦) or 161 mM trehalose (▲). Samples were lyophilized and stored at 30° C. followed by reconstitution with 0.9% benzyl alcohol to 100 mg/mL antibody in 20 mM histidine at pH 6 with isotonic (340 mM) and hypertonic (644 mM) sugar concentration.

FIG. **19** illustrates aggregation of rhuMAb E25 formulated at 25 mg/mL into 5 mM histidine at pH 6 with 85 mM sucrose (○); 85 mM trehalose (□); 161 mM sucrose (♦) or 161 mM trehalose (▲). Samples were lyophilized and stored at 50° C. followed by reconstitution with 0.9% benzyl alcohol to 100 mg/mL antibody in 20 mM histidine at pH 6 with isotonic (340 mM) and hypertonic (644 mM) sugar concentration.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

By "protein" is meant a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15–20 kD, preferably at least about 20 kD.

Examples of proteins encompassed within the definition herein include mammalian proteins, such as, e.g, growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α-1 -antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor , tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; tumor necrosis factor-α and -β; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as

aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-ell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-isted polypeptides.

The protein which is formulated is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc). "Essentially pure" protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" protein means a composition comprising at least about 99% by weight of protein, based on total weight of the composition.

In certain embodiments, the protein is an antibody. The antibody may bind to any of the above-mentioned molecules, for example. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the HER receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mol, p150,95, VLA-4, ICAM-1, VCAM and av/p3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; protein C etc.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature,* 256: 495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature,* 352:624–628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581–597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA,* 81:6851–6855 (1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature,* 321:522–525 (1986); Reichmann et al., *Nature,* 332:323–329 (1988); and Presta, *Curr. Op. Struct. Biol.,* 2:593–596 (1992). The humanized antibody includes a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

A "stable" formulation is one in which the protein therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in *Peptide and Protein Drug Delivery,* 247–301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. *Adv. Drug Delivery Rev.* 10: 29–90 (1993). Stability can be measured at a selected temperature for a selected time period. For rapid screening, the formulation may be kept at 40° C. for 2 weeks to 1 month, at which time stability is measured. Where the formulation is to be stored at 2–8° C., generally the formulation should be stable at 30° C. or 40° C. for at least 1 month and/or stable at 2–8° C. for at least 2 years. Where the formulation is to be stored at 30° C., generally the formulation should be stable for at least 2 years at 30° C. and/or stable at 40° C. for at least 6 months. For example, the extent of aggregation following lyophilization and storage can be used as an indicator of protein stability (see Examples herein). For example, a "stable" formulation may be one wherein less than about 10% and preferably less than about 5% of the protein is present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation following lyophilization and storage of the lyo-

philized formulation can be determined. For example, a "stable" lyophilized formulation may be one wherein the increase in aggregate in the lyoplilieed formulation is less than about 5% and preferably less than about 3%, when the lyophilized formulation is stored at 2–8° C. for at least one year. In other embodiments, stability of the protein formulation may be measured using a biological activity assay (see, e.g., Example 2 below).

A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein formulation in a diluent such that the protein is dispersed in the reconstituted formulation. The reconstituted formulation in suitable for administration (e.g. parenteral administration) to a patient to be treated with the protein of interest and, in certain embodiments of the invention, may be one which is suitable for subcutaneous administration.

By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 35OmOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

A "lyoprotectant" is a molecule which, when combined with a protein of interest, significantly prevents or reduces chemical and/or physical instability of the protein upon lyophilization and subsequent storage. Exemplary lyoprotectants include sugars such as sucrose or trehalose; an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a Iyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher sugar alcohols, e.g. glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and manmitol; propylene glycol; polyethylene glycol; Pluronics; and combinations thereof. The preferred lyoprotectant is a non-reducing sugar, such as trehalose or sucrose.

The lyoprotectant is added to the pre-lyophilized formulation in a "lyoprotecting amount" which means that, following lyophilization of the protein in the presence of the lyoprotecting amount of the lyoprotectant, the protein essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a reconstituted formulation. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

A "preservative" is a compound which can be added to the diluent to essentially reduce bacterial action in the reconstituted formulation, thus facilitating the production of a multi-use reconstituted formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, allyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol. The most preferred preservative herein is benzyl alcohol.

A "bulling agent" is a compound which adds mass to the lyophilized mixture and contributes to the physical structure of the lyophilized cake (e.g. facilitates the production of an essentially uniform lyophilized cake which maintains an open pore structure). Exemplary bulking agents include mannitol, glycine, polyethylene glycol and xorbitol. "Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

II. Modes for Carrying out the Invention

A. Protein Preparation

The protein to be formulated is prepared using techniques which are well established in the art including synthetic techniques (such as recombinant techniques and peptide synthesis or a combination of these techniques) or may be isolated from an endogenous source of the protein. In certain embodiments of the invention, the protein of choice is an antibody. Techniques for the production of antibodies follow.

(i) Polyclonal Antibodies.

Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining 1 mg or 1 μg of the peptide or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant. One month later the animals are boosted with ⅕ to ⅒ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal antibodies.

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59–103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51–63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies; Principles and Practice*, pp.59–103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256–262 (1993) and Pliuckthun, *Immunol. Revs.,* 130:151–188 (1992).

In a further embodiment, antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552–554 (1990). Clackson et al., *Nature,* 352:624–628 (1991) and Marks et al., *J. Mol Biol.,* 222:581–597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779–783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265–2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl. Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide-exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

(iii) Humanized and Human Antibodies.

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522–525 (1986); Riechmann et al., *Nature,* 332:323–327 (1988); Verhoeyen et al., *Science,* 239:1534–1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad Sci. USA,* 89:4285 (1992); Prestaetal., *J. Immnol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affmity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a

process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i. e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255–258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581–597 (1991)).

(iv) Bispecific Antibodies

Bispecific antibodies (BsAbs) are antibodies that have binding specificities for at least two different epitopes. Such antibodies can be derived from full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537–539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829 and in Traunecker et al., *EMBO J,* 10:3655–3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published March 3, 1994. For fuirther details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-inking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. The following techniques can also be used for the production of bivalent antibody fragments which are not necessarily bispecific. For example, Fab' fragments recovered from E. coli can be chemically coupled in vitro to form bivalent antibodies. See, Shalaby et al., *J. Exp. Med.,* 175:217–225 (1992).

Various techniques for making and isolating bivalent antibody fragments directly from recombinant cell culture have also been described. For example, bivalent heterodimers have been produced using leucine zippers. Kostelnyetal., *J. Immunol.,* 148(5):1547–1553 (1992). The leucine zipperpeptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444–6448 (1993) has provided an alternative mechanism for making bispecific/bivalent antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific/bivalent antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et aL, *J. Immunol.,* 152:5368 (1994).

B. Preparation of the Lyophilized Formulation

After preparation of the protein of interest as described above, a "pre-lyophilized formulation" is produced. The amount of protein present in the pre-lyophilized formulation is determined taking into account the desired dose volumes, mode(s) of administration etc. Where the protein of choice is an intact antibody (such as an anti-IgE or anti-HER2 antibody), from about 2 mg/mL to about 50 mg/mL, preferably from about 5 mg/mL to about 40 mg/mL and most preferably from about 20–30 mg/niL is an exemplary starting protein concentration. The protein is generally present in

solution. For example, the protein may be present in a pH-buffered solution at a pH from about 4–8, and preferably from about 5–7. Exemplary buffers include histidine, phosphate, Tris, citrate, succinate and other organic acids. The buffer concentration can be from about 1 mM to about 20 mM, or from about 3 mM to about 15 mM, depending, for example, on the buffer and the desired isotonicity of the formulation (e.g. of the reconstituted formulation). The preferred buffer is histidine in that, as demonstrated below, this can have lyoprotective properties. Succinate was shown to be another useful buffer.

The lyoprotectant is added to the pre-lyophilized formulation. In preferred embodiments, the lyoprotectant is a non-reducing sugar such as sucrose or trehalose. The amount of lyoprotectant in the pre-lyophilized formulation is generally such that, upon reconstitution, the resulting formulation will be isotonic. However, hypertonic reconstituted formulations may also be suitable. In addition, the amount of lyoprotectant must not be too low such that an unacceptable amount of degradation/aggregation of the protein occurs upon lyophilization. Where the lyoprotectant is a sugar (such as sucrose or trehalose) and the protein is an antibody, exemplary lyoprotectant concentrations in the pre-lyophilized formulation are from about 10 mM to about 400 mM, and preferably from about 30 mM to about 300 mM, and most preferably from about 50 mM to about 100 mM.

The ratio of protein to lyoprotectant is selected for each protein and lyoprotectant combination. In the case of an antibody as the protein of choice and a sugar (e.g., sucrose or trehalose) as the lyoprotectant for generating an isotonic reconstituted formulation with a high protein concentration, the molar ratio of lyoprotectantto antibody may be from about 100 to about 1500 moles lyoprotectant to 1 mole antibody, and preferably from about 200 to about 1000 moles of lyoprotectant to 1 mole antibody, for example from about 200 to about 600 moles of lyoprotectant to 1 mole antibody.

In preferred embodiments of the invention, it has been found to be desirable to add a surfactant to the pre-lyophilized formulation. Alternatively, or in addition, the surfactant may be added to the lyophilized formulation and/or the reconstituted formulation. Exemplary surfactants include nonionic surfactants such as polysorbates (e.g. polysorbates 20 or 80); poloxamers (e.g. poloxamer 188); Triton; sodium dodecyl sulfate (SDS); sodium laurel sulfate; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetaine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauroamidopropyl-, cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palnidopropyl-, or isostearamidopropyl-betaine (e.g lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-taurate; and the MONAQUAT™ series (Mona Industries, Inc., Paterson, N.J.), polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (e.g. Pluronics, PF68 etc). The amount of surfactant added is such that it reduces aggregation of the reconstituted protein and minimizes the formation of particulates after reconstitution. For example, the surfactant may be present in the pre-lyophilized formulation in an amount from about 0.001–0.5%, and preferably from about 0.005–0.05%.

In certain embodiments of the invention, a mixture of the lyoprotectant (such as sucrose or trehalose) and a bulking agent (e.g. mannitol or glycine) is used in the preparation of the pre-lyophilization formulation. The bulking agent may allow for the production of a uniform lyophilized cake without excessive pockets therein etc.

Other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980) may be included in the pre-lyophilized formulation (and/or the lyophilized formulation and/or the reconstituted formulation) provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include; additional buffering agents; preservatives; co-solvents; antioxidants including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g. Zn-protein complexes); biodegradable polymers such as polyesters; and/or salt-forming counterions such as sodium.

The formulation herein may also contain more than one protein as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect the other protein. For example, it may be desirable to provide two or more antibodies which bind to the HER2 receptor or IgE in a single formulation. Furthermore, anti-HER2 and anti-VEGF antibodies may be combined in the one formulation. Such proteins are suitably present in combination in amounts that are effective for the purpose intended.

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to, or following, lyophilization and reconstitution. Alternatively, sterility of the entire mixture may be accomplished by autoclaving the ingredients, except for protein, at about 120° C. for about 30 minutes, for example.

After the protein, lyoprotectant and other optional components are mixed together, the formulation is lyophilized. Many different freeze-dryers are available for this purpose such as Hull50™ (Hull, USA) or GT20™ (Leybold-Heraeus, Germany) freeze-dryers. Freeze-drying is accomplished by freezing the formulation and subsequently subliming ice from the frozen content at a temperature suitable for primary drying. Under this condition, the product temperature is below the eutectic point or the collapse temperature of the formulation. Typically, the shelf temperature for the primary drying will range from about −30 to 25° C. (provided the product remains frozen during primary drying) at a suitable pressure, ranging typically from about 50 to 250 mTorr. The formulation, size and type of the container holding the sample (e.g., glass vial) and the volume of liquid will mainly dictate the time required for drying, which can range from a few hours to several days (e.g. 40–60hrs). A secondary drying stage may be carried out at about 0–40° C., depending primarily on the type and size of container and the type of protein employed. However, it was found herein that a secondary drying step may not be necessary. For example, the shelf temperature throughout the entire water removal phase of lyophilization may be from about 15–30° C. (e.g., about 20° C.). The time and pressure required for secondary drying will be that which produces a suitable lyophilized cake, dependent, e.g., on the temperature and other parameters. The secondary drying time is dictated by the desired residual moisture level in the product and typically takes at least about 5 hours (e.g. 10–15 hours). The pressure may be the same as that employed during the primary drying step. Freeze-drying conditions can be varied depending on the formulation and vial size.

In some instances, it may be desirable to lyophilize the protein formulation in the container in which reconstitution of the protein is to be carried out in order to avoid a transfer step. The container in this instance may, for example, be a 3, 5, 10, 20, 50 or 100 cc vial.

As a general proposition, lyophilization will result in a lyophilized formulation in which the moisture content thereof is less than about 5%, and preferably less than about 3%.

C. Reconstitution of the Lyophilized Formulation

At the desired stage, typically when it is time to administer the protein to the patient, the lyophilized formulation may be reconstituted with a diluent such that the protein concentration in the reconstituted formulation is at least 50 mg/mL, for example from about 50 mg/mL to about 400 mg/mL, more preferably from about 80 mg/mL to about 300 mg/mL, and most preferably from about 90 mg/mL to about 150 mg/mL. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where subcutaneous delivery of the reconstituted formulation is intended. However, for other routes of administration, such as intravenous administration, lower concentrations of the protein in the reconstituted formulation may be desired (for example from about 5–50 mg/mL, or from about 10–40 mg/mL protein in the reconstituted formulation). In certain embodiments, the protein concentration in the reconstituted formulation is significantly higher than that in the pre-lyophilized formulation. For example, the protein concentration in the reconstituted formulation may be about 2–40 times, preferably 3–10 times and most preferably 3–6 times (e.g. at least three fold or at least four fold) that of the pre-lyophilized formulation.

Reconstitution generally takes place at a temperature of about 25° C. to ensure complete hydration, although other temperatures may be employed as desired. The time required for reconstitution will depend, e.g., on the type of diluent, amount of excipient(s) and protein. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. The diluent optionally contains a preservative. Exemplary preservatives have been described above, with aromatic alcohols such as benzyl or phenol alcohol being the preferred preservatives. The amount of preservative employed is determined by assessing different preservative concentrations for compatibility with the protein and preservative efficacy testing. For example, if the preservative is an aromatic alcohol (such as benzyl alcohol), it can be present in an amount from about 0.1–2.0% and preferably from about 0.5–1.5%, but most preferably about 1.0–1.2%.

Preferably, the reconstituted formulation has less than 6000 particles per vial which are >10 μm m size.

D. Administration of the Reconstituted Formulation

The reconstituted formulation is administered to a mammal in need of treatment with the protein, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

In preferred embodiments, the reconstituted formulation is administered to the mammal by subcutaneous (i.e. beneath the skin) administration. For such purposes, the formulation may be injected using a syringe. However, other devices for administration of the formulation are available such as injection devices (e.g. the Inject-ease™ and Genject™ devices); injector pens (such as the GenPen™); needleless devices (e.g. MediJector™ and BioJector™); and subcutaneous patch delivery systems.

The appropriate dosage ("therapeutically effective amount") of the protein will depend, for example, on the condition to be treated, the severity and course of the condition, whether the protein is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the protein, the type of protein used, and the discretion of the attending physician. The protein is suitably administered to the patient at one time or over a series of treatments and may be administered to the patient at any time from diagnosis onwards. The protein may be administered as the sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question.

Where the protein of choice is an antibody, from about 0.1–20 mg/kg is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques.

In the case of an anti-HER2 antibody, a therapeutically effective amount of the antibody may be administered to treat or prevent cancer characterized by overexpression of the HER2 receptor. It is contemplated that a reconstituted formulation of the anti-HER2 antibody may be used to treat breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon and/or bladder cancer. For example, the anti-HER2 antibody may be used to treat ductal carcinoma in situ (DCIS). Exemplary dosages of the anti-HER2 antibody are in the range 1–10 mg/kg by one or more separate administrations.

Uses for an anti-IgE formulation include the treatment or prophylaxis of IgE-mediated allergic diseases, parasitic infections, interstitial cystitis and asthma, for example. Depending on the disease or disorder to be treated, a therapeutically effective amount (e.g. from about 1–15 mg/kg) of the anti-IgE antibody is administered to the patient.

E. Articles of Manufacture

In another embodiment of the invention, an article of manufacture is provided which contains the lyophilized formulation of the present invention and provides instructions for its reconstitution and/or use. The article of manufacture comprises a container. Suitable containers include, for example, bottles, vials (e.g. dual chamber vials), syringes (such as dual chamber syringes) and test tubes. The container may be formed from a variety of materials such as glass or plastic. The container holds the lyophilized formulation and the label on, or associated with, the container may indicate directions for reconstitution and/or use. For example, the label may indicate that the lyophilized formulation is reconstituted to protein concentrations as described above. The label may further indicate that the formulation is useful or intended for subcutaneous administration. The container holding the formulation may be a multi-use vial, which allows for repeat administrations (e.g from 2–6 administrations) of the reconstituted formulation. The article of manufacture may further comprise a second container comprising a suitable diluent (e.g. BWFI). Upon mixing of the diluent and the lyophilized formulation, the final protein concentration in the reconstituted formulation will generally be at least 50 mg/mL. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. All literature citations are incorporated by reference.

EXAMPLE 1

Anti-Her2 Formulation

Overexpression of the HER2 proto-oncogene product (P_{185}^{HER2}) has been associated with a variety of aggressive human malignancies. The murine monoclonal antibody known as muMAb4D5 is directed against the extracellular domain (ECD) of $p185^{HER2}$. The muMAb4D5 molecule has been humanized in an attempt to improve its clinical efficacy by reducing immunogenicity and allowing it to support human effector functions (see WO 92/22653). This example describes the development of a lyophilized formulation comprising full length humanized antibody huMAb4D5–8 described in WO 92/22653.

In the development of a lyophilized formulation, excipients and buffers are initially screened by measuring the stability of the protein after lyophilization and reconstitution. The lyophilized protein in each formulation is also subjected to accelerated stability studies to determine the potential stability of the protein over its shelf-life. These accelerated studies are usually performed at temperatures above the proposed storage conditions and the data are then used to estimate the activation energy for the degradation reactions assuming Arrhenius kinetics (Cleland et al., *Critical Reviews in Therapeutic Drug Carrier Systems* 10(4): 307–377 (1993)). The activation energy is then used to calculate the expected shelf-life of the protein formulation at the proposed storage conditions.

In early screening studies, the stability of several lyophilized recombinant humanized anti-HER2 antibody (rhuMAb HER2) formulations was investigated after incubation at 5° C. (proposed storage condition) and 40° C. (accelerated stability condition). In the liquid state, rhuMAb HER2 was observed to degrade by deamidation (30 Asn of light chain) and isoaspartate formation via a cyclic imide intermediate, succinimide (102 Asp of heavy chain). The deamidation was minimized at pH 5.0 resulting in degradation primarily at the succinimide. At pH 6.0, slightly greater deamidation was observed in the liquid protein formulation. The lyophilized formulations were therefore studied with: (a) 5 or 10 mM succinate buffer, pH 5.0 or (b) 5 or 10 mM histidine buffer, pH 6.0. Both buffers contained the surfactant, polysorbate 20 (Tween 20™), which was employed to reduce the potential for aggregation of the reconstituted protein and minimize the formation of particulates after reconstitution. These buffers were used with and without various sugars. The protein was formulated in the buffer at 5.0, 21.0 or 25.0 mg/mL. These formulations were then lyophilized and assessed for protein stability after 2 weeks at 5° C. and 40° C. In the lyophilizer, the vials were frozen at a shelf temperature of −55° C. for approximately 5 hours followed by primary drying at a shelf temperature of 5° C. and 150 mTorr for 30 hours, and drying to 1–2% residual moisture was achieved with secondary drying at a shelf temperature of 20° C. for 10 hours. The major degradation route for this protein upon lyophilization was aggregation, and therefore the protein stability was assessed by native size exclusion chromatography to measure the recovery of intact native protein (% intact protein in Table 2 below).

The stabilizing effects of various lyoprotectant sugars on lyophilized protein was measured in 10 mM sodium succinate, pH 5.0 (Table 2). At high sugar concentrations (250–275 mM) and low protein concentration (5.0 mg/mL), trehalose and lactose stabilized the protein against aggregation for the lyophilized protein stored for 2 weeks at 40° C. However, lactose, a reducing sugar, was observed to react with the protein over longer term storage at 40° C. The formulations at 5.0 mg/mL protein containing either sorbitol or mannitol yielded aggregated protein after storage at 40° C. for 2 weeks. At the higher protein concentration (21.0 mg/mL), formulations comprising mannitol, or mannitol in combination with sorbitol or glycine, contained aggregated protein after lyophilization and storage at both conditions. In contrast, trehalose and sucrose prevented aggregation at both storage conditions.

The 250 mM trehalose and 250 mM lactose formulations were assessed for long term stability. After 9 months at 40° C. or 12 months at 5° C., there was no change in the % intact protein for the trehalose formulation. For the lactose formulation, the % intact protein remained constant (same as initial) after 3 months at 40° C. or 6 months at 25° C. The trehalose formulation could be stored at controlled room temperature (15–30° C.) for 2 years without a significant change in % intact protein.

The 10 mM histidine, pH 6.0 formulation with mannitol contained less aggregated protein after storage at 40° C. for 2 weeks than the 10 mM succinate formulation, pH 5.0 with mannitol. This result may be related to some stabilizing effect contributed by histidine alone. After storage at 40° C. for 2 weeks, there was, however, significant aggregation for histidine alone or histidine/mannitol formulations. The addition of sucrose at an equal mass to mannitol (10 mg/mL of each) in the histidine formulation stabilized the protein against aggregation for both storage conditions. The use of glycine with mannitol did not improve the protein stability, while the sucrose/glycine formulation provided the same stability as the sucrose/mannitol formulation. These results further indicated that sucrose was useful for preventing aggregation of the lyophilized protein during storage.

TABLE 2

Composition Prior to Lyophilization		% Intact Protein[a]		
[Protein][b] (mg/mL)	Formulation	Liquid (5° C.)	Lyophilized (2 wk, 5° C.)	Lyophilized (2 wk, 40° C.)
	10 mM sodium succinate pH 5.0			
5.0	275 mM trehalose, 0.01% Tween 20 ™	98.9	99.1	98.9
5.0	275 mM lactose, 0.01% Tween 20 ™	96.8	96.5	96.6
5.0	275 mM sorbitol, 0.01% Tween 20 ™	99.4	99.3	95.4
5.0	250 mM mannitol, 0.01% Tween 20 ™	100.0	99.9	98.8
5.0	250 mM trehalose, 0.01% Tween 20 ™	100.0	99.9	100.0
5.0	250 mM lactose, 0.01% Tween 20 ™	100.0	100.0	100.0
21.0	250 mM trehalose, 0.2% Tween 20 ™	99.3	99.1	99.1
21.0	250 mM sucrose, 0.2% Tween 20 ™	99.6	99.6	99.7
21.0	250 mM mannitol, 0.01% Tween 20 ™	100.0	94.6	94.0
21.0	188 mM mannitol/63 mM sorbitol, 0.01% Tween 20 ™	99.8	98.6	96.5
21.0	250 mM mannitol/25 mM glycine, 0.01% Tween 20 ™	99.5	96.5	96.4
	10 mM histidine pH 6.0			
21.0	No sugar, 0.01% Tween 20 ™	100.0	99.9	98.9
21.0	54.9 mM mannitol, 0.01% Tween 20 ™	100.0	99.9	99.2
21.0	29.2 mM sucrose/266.4 mM glycine, 0.01% Tween 20 ™	100.0	100.0	99.6

TABLE 2-continued

Composition Prior to Lyophilization		% Intact Protein[a]		
[Protein][b] (mg/mL)	Formulation	Liquid (5° C.)	Lyophilized (2 wk, 5° C.)	Lyophilized (2 wk, 40° C.)
21.0	54.9 mM mannitol/266.4 mM glycine, 0.01% Tween 20 ™	100.0	99.8	98.9
21.0	54.9 mM mannitol/29.2 mM sucrose, 0.01% Tween 20 ™	99.8	100.0	99.7

[a]The fraction of intact protein was measured by native size exclusion HPLC and the peak area of the native protein relative to the total peak area including aggregates (TSK3000 SW XL column, TosoHaas, with a flow rate of 1.0 mL/min; elution with phosphate buffered saline; detection at 214 and 280 nm). The protein formulations were analyzed before lyophilization (liquid, 5° C.) and after lyophilization and storage at 5° C. or 40° C. for 2 weeks.
[b]Formulations containing 5 mg/mL protein were reconstituted with distilled water (20 mL, 5.0 mg/mL protein), and formulations containing 21 mg/mL protein were reconstituted with bacteriostatic water for injection (BWFI, 0.9% benzyl alcohol; 20 mL, 20 mg/mL protein).

The delivery of a high protein concentration is often required for subcutaneous administration due to the volume limitations (≦1.5 mL) and dosing requirements (≧100 mg). However, high protein concentrations (≧50 mg/mL) are often difficult to achieve in the manufacturing process since at high concentrations, the protein has a tendency to aggregate during processing and becomes difficult to manipulate (e.g. pump) and sterile filter. Alternatively, the lyophilization process may provide a method to allow concentration of the protein. For example, the protein is filled into vials at a volume (Vf) and then lyophilized. The lyophilized protein is then reconstituted with a smaller volume (Vr) of water or preservative (e.g. BWFI) than the original volume (e.g. Vr=0.25 Vf) resulting in a higher protein concentration in the reconstituted solution. This process also results in the concentration of the buffers and excipients. For subcutaneous administration, the solution is desirably isotonic.

The amount of trehalose in the lyophilized rhuMAb HER2 was reduced to produce an isotonic solution upon reconstitution to yield 100 mg/mL protein. The stabilizing effect of trehalose was determined as a function of concentration for 5 mM sodium succinate, pH 5.0 and 5 mM histidine, pH 6.0 at 25.0 mg/mL protein (Table 3). Attrehalose concentrations from 60 to 200 mM, there was no significant aggregation after incubation of the lyophiized protein for 4 weeks at 40° C. These formulations were reconstituted with 20 mL of bacteriostatic water for injection (BWFI, USP, 0.9% benzyl alcohol). Reconstitution of the 50 mM trehalose formulation (5 mM sodium succinate) with 4 mL of BWFI (100 mg/mL protein) after incubation for 4 weeks at 40° C. yielded a slight increase in aggregate formation. The preserved reconstituted formulations provided the advantage of multiple withdrawals from the same vial without sterility concerns. When sterile needles are used, these formulations would then allow for several doses from a single vial.

TABLE 3

Composition Prior to Lyophilization		% Intact Protein[a]		
[Protein] (mg/mL)	Formulation	Liquid (5° C.)	Lyophilized (4 wk, 5° C.)	Lyophilized (4 wk, 40° C.)
	5 mM sodium succinate pH 5.0			
25.0	50 mM trehalose, 0.01% Tween 20 ™[b]	100.00	100.0	99.5
25.0	60 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.9
25.0	60 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.2
25.0	100 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.7
25.0	150 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.8
25.0	200 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	100.0
	5 mM histidine pH 6.0			
25.0	38.4 mM mannitol/20.4 mM sucrose, 0.01% Tween 20 ™	100.0	100.0	99.3
25.0	38.4 mM mannito1/20.4 mM sucrose, 0.01% Tween 20 ™[c]	100.0	100.0	99.4
25.0	60 mM trehalose, 0.01% Tween 20 ™[d]	100.0	100.0	99.8
25.0	60 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.4
25.0	100 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	99.6
25.0	150 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	100.0
25.0	200 mM trehalose, 0.01% Tween 20 ™	100.0	100.0	100.0

[a]The fraction of intact protein was measured by native size exclusion HPLC and defined as the peak area of the native protein relative to the total peak area including aggregates (TSK3000 SW XL column, TosoHaas, with a flow rate of 1.0 mL/min; elution with phosphate buffered saline; detection at 214 and 280 nm). The protein formulations were analyzed before lyophilization (liquid, 5° C.) and after lyophilization and storage at 5° C. or 40° C. for 4 weeks. Formulations were reconstituted with bacteriostatic water for injection (BWFI, USP, 0.9% w/w benzyl alcohol; 20 mL, 22 mg/mL protein).

TABLE 3-continued

Composition Prior to Lyophilization		% Intact Protein[a]		
[Protein] (mg/mL)	Formulation	Liquid (5° C.)	Lyophilized (4 wk, 5° C.)	Lyophilized (4 wk, 40° C.)

[b]Reconstituted with 4 mL of BWFI (0.9% benzyl alcohol) to yield 100 mg/mL protein.
[c]Reconstituted with 4 mL of BWFI (1.1% benzyl alcohol) to yield 100 mg/mL protein.
[d]Sample incubated for 2 weeks at 5° C. or 40° C. and then reconstituted with 20 mL of BWFI (0.9% benzyl alcohol) to yield 22 mg/mL protein.

Currently, rhuMAb HER2 is under investigation as a therapeutic for the treatment of breast cancer. The protein is dosed to patients at 2 mg/kg on a weekly basis. Since the average weight of these patients is 65 kg, the average weekly dose is 130 mg of rhuMAb HER2. For subcutaneous administration, injection volumes of 1.5 mL or less are well tolerated and, therefore, the protein concentration for a weekly subcutaneous administration of rhuMAb HER2 maybe approximately 100 mg/mL (130 mg average dose/1.5 mL). As mentioned above, this high protein concentration is difficult to manufacture and maintain in a stable form. To achieve this high protein concentration, rhuMAb HER2 formulated in: (a) 5 mM sodium succinate, pH 5.0 or (b) 5 mM histidine, pH 6.0, was lyophilized at 25 mg/mL protein in 60 mM trehalose, 0.01% Tween 20™. The lyophilization was performed by filling 18 mL of the protein formulation into 50 ° C. vials. In the lyophilizer, the vials were frozen at a shelf temperature of −55° C. for approximately 5 hours followed by primary drying at a shelf temperature of 5° C. and 150 mTorr for 30 hours, and drying to 1–2% residual moisture was achieved with secondary drying at a shelf temperature of 20° C. for 10 hours. Thermocouples placed in vials containing the placebo (formulation without protein) indicated that the product in the vials was maintained below −10° C. throughout primary drying. Sequential stoppering studies during the lyophilization revealed that the residual moisture after primary drying was usually less than 10%.

The lyophilized protein was then reconstituted with either 4 or 20 mL of BWFI (0.9 or 1.1% benzyl alcohol) to yield concentrated protein solutions:

(a) 4 mL: 102 mg/mL rhuMAb HER2, 245 mM trehalose, 21 mM sodium succinate, pH 5.0 or 21 mM histidine, pH 6.0, 0.04% Tween 20™;

(b) 20 mL: 22 mg/mL rhuMAb HER2, 52 mM trehalose, 4 mM sodium succinate, pH 5.0 or 4 mM histidine, pH 6.0, 0.009% Tween 20™.

After storage of the lyophilized formulations for 4 weeks at 40° C. and reconstitution to 22 mg/mL protein, the amount of aggregated protein appeared to increase slightly with decreasing trehalose concentration. The stability of the lyophilized protein was not affected by the volume of reconstitution. As shown in FIG. 1, the amount of intact protein after incubation of the lyophilized protein at 40° C. was the same for the 60 mM trehalose, 5 mM sodium succinate, pH 5.0, 0.01% Tween 2OTM formulation reconstituted with either 4 or 20 mL of BWFI.

The results shown in Table 3 suggested that there may be a relationship between the trehalose concentration and the protein stability. To further assess this relationship, the formulations containing different concentrations of trehalose formulated in either sodium succinate or histidine were incubated for 91 days at 40° C. The stability was then measured as a function of the trehalose to protein molar ratio for each concentration of trehalose. As shown in FIG. 2, the protein stability clearly decreased with decreasing trehalose concentration for both formulations. There was no apparent difference between the two buffers, succinate and histidine, in these formulations suggesting that the primary stabilizer under these conditions is trehalose. In addition, the observed decrease in intact protein for both these formulations would be acceptable even at the low trehalose concentration for a formulation that is stored at 2–8° C. throughout its shelf-life. However, if controlled room temperature (30° C. maxmimum temperature) stability is required, higher trehalose concentrations (≧600:1 trehalose:protein) may be needed depending on the stability specifications for the product (i.e. the specification for the amount of intact protein remaining after 2 years of storage). Typically, a controlled room temperature storage condition would require stability for 6 months at 40° C. which is equivalent to storage at 30° C. for 2 years.

As shown in FIG. 3, the 250 mM trehalose formulation was unchanged after 6 months at 40° C. while both the 60 mM trehalose formulations were less stable. The 60 mM trehalose formulations may then require refrigerated storage if the product specification at the end of its shelf-life is, for example, >98% intact protein by native size exclusion chromatography.

In the previous screening study, sucrose was also observed to prevent aggregation of rhuMAb HER2 after lyophilization and subsequent storage. To achieve isotonic solutions after reconstitution for subcutaneous administration (approximately four-fold concentration of formulation components and protein), the sucrose concentration must be reduced significantly. The equal mass concentration of sucrose and mannitol (bulking agent) used in the screening studies prevented aggregation of the protein. A lower concentration of sucrose and mannitol (equal mass concentrations) was chosen as a potential subcutaneous formulation of rhuMAb HER2. The protein solution (25 mg/mL protein, 5 mM histidine, pH 6.0, 38.4 mM (7 mg/mL) mannitol, 20.4 mM (7 mg/nL) sucrose, 0.01% Tween 20Tm) was lyophilized in the same manner as the 60 mM trehalose formulation except that the primary drying cycle was extended to 54 hours. After 4 weeks at 40° C., there was a slight increase in the amount of aggregates after reconstitution with 4.0 or 20.0 mL of BWFI (Table 3). The amount of aggregated protein was the same for reconstitution at 22 or 100 mg/mL protein (FIG. 4). Like the 60 mM trehalose formulations, the mannitol/sucrose formulation yielded less intact protein over time at 40° C. The molar ratio of sucrose to protein for this formulation was 120 to 1, indicating that the mannitoVsucrose combination may be more effective than trehalose alone at the same molar ratio of stabilizing sugar (FIGS. 2 and 4).

In the previous examples, the stability of the lyophilized rhuMAb HER2 formulations was determined as a function of temperature. These studies demonstrated that the trehalose and mannitol/sucrose formulations prevented degradation of the protein in the lyophilized state at high temperatures (40° C.). However, these experiments did not address the stability of the protein after reconstitution and storage. Once reconstituted with BWFI, the lyophilized rhuMAb HER2 formulations may be used for several administrations of the drug. In particular, the vial configuration (450 mg rhuMAb HER2) was designed to provide three doses to the average patient (130 mg rhuMAb HER2 per dose). Since the

drug is dosed weekly, the vial maybe stored at least three weeks after reconstitution. To assure that the rhuMAb HER2 remained stable after reconstitution, stability studies on the reconstituted rhuMAb HER2 formulations were performed at 5° C. and 25° C.

For subcutaneous administration, the formulations were reconstituted to 100 mg/mL (4 mL BWFI). At this high protein concentration, the protein may be more susceptible to aggregation than the intravenous dosage form that was reconstituted to 22 mg/mL protein (20 mL BWFI). The four rhuMAb HER2 formulations from the previous example were assessed for aggregation (loss of intact protein). As shown in Tables 4 through 6, there was no difference in stability for formulations reconstituted at 22 and 100 mg/mL protein. Furthermore, these formulations maintained the protein completely intact for up to 90 days at 5° C. and 30 days at 25° C., indicating that the reconstituted protein could be stored refrigerated for at least 90 days. Unlike the lyopbilized protein stability in the previous example, the trehalose concentration in the formulation did not affect the protein stability (Table 7).

TABLE 4

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 25 mg/mL protein in 5 mM sodium succinate, pH 5.0, 60 mM trehalose, 0.01% Tween 20 ™

Time	% Intact Protein			
	22 mg/mL protein		100 mg/mL protein	
(days)	5° C.	25° C.	5° C.	25° C.
0	99.9	99.9	99.7	99.7
14	ND	100.0	ND	100.0
30	100.0	100.0	100.0	100.0
91	99.8	ND	100	ND

The samples were reconstituted with 4.0 or 20.0 mL of BWFI (1.1% or 0.9% benzyl alcohol), and then stored at 5° C. or 25° C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

TABLE 5

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 25 mg/mL protein in 5 mM histidine, pH 6.0, 60 mM trehalose, 0.01% Tween 20 ™

Time	% Intact Protein			
	22 mg/mL protein		100 mg/mL protein	
(days)	5° C.	25° C.	5° C.	25° C.
0	100.0	100.0	100.0	100.0
14	ND	100.0	ND	100.0
31	99.3	99.7	100.0	100.0
61	100.0	ND	ND	ND

The samples were reconstituted with 4.0 or 20.0 mL of BWFI (1.1% or 0.9% benzyl alcohol), and then stored at 5° C. or 25° C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

TABLE 6

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 25 mg/mL protein in 5 mM histidine, pH 6.0, 38.4 mM mannitol, 20.4% mM sucrose, 0.01% Tween 20 ™

Time	% Intact Protein			
	22 mg/mL protein		100 mg/mL protein	
(days)	5° C.	25° C.	5° C.	25° C.
0	99.7	99.7	99.8	99.8
14	ND	100.0	ND	99.8
31	100.0	100.0	100.0	100.0
92	100.0	ND	100.0	ND

The samples were reconstituted with 4.0 or 20.0 mL of BWFI (1.1% or 0.9% benzyl alcohol), and then stored at 5° C. or 25° C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

TABLE 7

Stability of the reconstituted formulations for rhuMAb HER2 lyophilized at 21 mg/mL protein in 10 mM sodium succinate, pH 5.0, 250 mM trehalose, 0.2% Tween 20 ™

Time	% Intact Protein 21 mg/mL protein	
(days)	5° C.	25° C.
0	99.8	99.8
14	ND	100.0
31	99.9	99.4
92	99.8	ND

The samples were reconstituted with 20.0 mL of BWFI (0.9% benzyl alcohol), and then stored at 5° C. or 25° C. The % intact protein was defined as the fraction of native peak area as measured by native size exclusion chromatography. ND = not determined.

As mentioned previously, the major degradation route for rhuMAb HER2 in aqueous solutions is deamidation or succinimide formation. The loss of native protein due to deamidation or succinimide formation was assessed for the four reconstituted rhuMAb HER2 formulations.

Analysis of rhuMAb HER2 deamidation and succinimide formation was performed using cation exchange chromatography. A Bakerbond Wide-Pore Carboxy Sulfon (CSX) column (4.6×250 mm) was operated at a flow rate of 1 mL/min. The mobile phase buffers were (A) 0.02 M sodium phosphate, pH 6.9, and (B) 0.02 M sodium phosphate, pH 6.9, 0.2 M NaCl. The chromatography was then performed at 40° C. as follows:

TABLE 8

Time (min)	% Buffer B
0	10
55	45
57	100
62	100
62.1	10
63	10

Peak elution was monitored at 214 nm and 75 μg of protein was loaded for each analysis.

Again, there were no differences in stability of the formulations reconstituted at 22 and 100 mg/mL protein (FIGS. 5 through 7). The protein degradation was more rapid at 25° C. than 5° C. for each formulation, and the rate of degradation was comparable for all the formulations stored at 5° C. The formulations containing histidine underwent a slightly greater rate of degradation at 25 C than the succinate

formulations. The amount of trehalose in the formulation did not affect the degradation rate at either temperature (FIGS. 5 and 8). These results indicated that these four formulations provide an acceptable rate of degradation under refrigerated storage conditions (5° C.) for the intended period of use (30 days after reconstitution with BWFI).

Multi-use formulations should pass preservative efficacy testing as described by the US Pharmacopeia (USP) for use in the United States. The rhuMAb HER2 lyophilized formulation consisting of 25 mg/mL protein, 5 mM histidine, pH 6.0, 60 mM trehalose, 0.01% Tween 20™ was reconstituted with 20 mL of benzyl alcohol at concentrations between 0.9 and 1.5% w/w. For concentrations at or above 1.3% w/w, the reconstituted solution became cloudy after overnight incubation at room temperature (~25° C.). Reconstitution with the standard BWFI solution (0.9% benzyl alcohol) resulted in a solution that did not consistently pass the preservative challenge tests. However, reconstitution with 1.0 or 1.1% benzyl alcohol was both compatible with the formulation and passed the preservative challenge testing. The manufacturer's specifications for the solution required a range of +10%, and therefore, the lyophilized formulations are reconstituted with 1.1% benzyl alcohol (1.1±0.1%).

A single step lyophilization cycle for the rhuMAb HER2 formulation was developed . In the single step lyophilization cycle, rhuMAb HER2 at 25 mg/mL, 60 mM trehalose, 5 mM histidine pH 6 and 0.01% polysorbate 20 was lyophilized at a shelf temperature of 20° C., and a pressure of 150 mTorr. After 47 hours, the residual moisture content of the lyophilized cake was less than 5%. This lyophilization cycle is considered to be useful in that it simplifies the manufacturing process, by eliminating the secondary drying step.

EXAMPLE 2

Anti-IgE Formulation

IgE antibodies bind to specific high-affinity receptors on mast cells, leading to mast cell degranulation and release of mediators, such as histamine, which produce symptoms associated with allergy. Hence, anti-IgE antibodies that block binding of IgE to its high-affinity receptor are of potential therapeutic value in the treatment of allergy. These antibodies must also not bind to IgE once it is bound to the receptor because this would trigger histamine release. This example describes the development of a lyophilized formulation comprising full length humanized anti-IgE antibody MaE11 described in Presta et al. *J. Immunology,* 151: 2623–2632 (1993).

Materials:

Highly purified rhuMAb E25 (recombinant humanized anti-IgE antibody MaE11) which did not contain Tween 20™ was used in the formulations described below. Spectra/Por 7 dialysis membranes were purchased from Spectrum (Los Angeles, Calif.). All other reagents used in this study were obtained from commercial sources and were of analytical grade. Formulation buffers and chromatography mobile phase were prepared by mixing the appropriate amount of buffer and salt with Milli-Q water in a volumetric flask.

Formulation:

E25 S Sepharose pool was dialyzed into formulation buffers as specified. Dialysis was accomplished by a minimum of 4×2L buffer exchanges over a 48 hour period at 2–8° C. Following dialysis, lyoprotectant was added at a isotonic concentration to some of the formulations as required. Protein concentration following dialysis was determined by UV spectroscopy using a molar absorptivity of 1.60. The dialyzed protein was diluted to the predetermined formulation concentration with an appropriate formulation buffer, sterile filtered using a 0.22 μm Millex-GV filter (Millipore) and dispensed into pre-washed and autoclaved glass vials. The vials were fitted with siliconized teflon lyophilization stoppers and lyophlized using the following conditions: the E25 formulation was frozen to −55° C. at 80° C./hour and the vial content was kept frozen for 4 hours. The shelf temperature was ramped to 25° C. at 10° C./hour for primary drying. Primary drying was carried out at 25° C., 50μ chamber vacuum pressure for 39 hours such that the residual moisture of the lyophilized cake was 1–2%. Following lyophilization, a vial of each formulation was removed for t=0 analysis and the remaining vials were held at various temperatures which include −70° C., 2–8° C., 25° C., 30° C. (controlled room temperature) 40° C. and 50° C.

Chromatography:

Native size exclusion chromatography was carried out on a Bio-Rad Bio-Select™ SEC 250-5 column (300×7.8 mm). The column was equilibrated and ran in PBS at a flow rate of 0.5 mL/min using a Hewlett Packard 1090L HPLC equipped with a diode array detector. Molecular weight standards (Bio-Rad, Inc.) consisting of thyroglobulin (670 kd), gamma-globulin (158 kd), ovalbumin (44 kd), and cyanocobalamin (1.35 kd) were used to calibrate the column. The sample load was 25 μg and protein was detected by monitoring the UV absorption at 214 nm using Turbochrom 3 software (PE Nelson, Inc).

Hydrophobic Interaction Chromatography:

$F(ab')_2$ fragments of the E25 antibody were chromatographed using a TosoHaas Butyl-NPR column (3.5×4.6 mm) and a Hewlett Packard 1090L HPLC equipped with a diode array detector. Elution buffer A was: 20 mM Tris, 2 M ammonium sulfate, 20% (v/v) glycerol, pH 8.0 while elution buffer B was: 20 mM Tris, 20% (v/v) glycerol, pH 8.0. The column was equilibrated with 10% elution buffer B at a flow rate of 1.0 mL/min for a minimum of 20 minutes. The sample load was 5 μg and protein was detected by monitoring the UV absorption at 214 nm using Turbochrom 3 data acquisition software (PE Nelson, Inc). Following injection of the sample, the column was maintained at 10% buffer B for 1 minute followed by a linear gradient of from 10% to 62% buffer B in 20 minutes. The column was washed with 100% buffer B for 5 minutes and re-equilibrated with 10% buffer B for a minimum of 20 minutes between successive sample injections.

Antibody Binding Activity:

IgE receptor binding inhibition assay (IE25 :2) was carried out as described in Presta et al., supra, on samples diluted to 20 μg/mL and 30 μg/mL in assay diluent (phosphate buffered saline, 0.5% BSA, 0.05% polysorbate 20, 0.01% Thimerosol). Each dilution was then assayed in triplicate and the results were multiplied by an appropriate dilution factor to yield an active concentration. The results from 6 assays were averaged. The assay measures the ability of rhuMAb E25 to competitively bind to IgE and thereby prevent IgE from binding to its high affinity receptor which is immobilized to an ELISA plate. The results are divided by the antibody concentration as determined by UV absorption spectroscopy and reported as a specific activity. Previous experiments have shown that this assay is stability indicating.

Particulate Assay:

Reconstituted vials of lyophilized rhuMAb E25 were pooled to achieve a volume of approximately 7 mL. A count of the number of particles of size ranging from 2 to 80 μm present in 1 mL of sample was determined using a Hiac/Royco model 8000 counter. The counter was first washed with 1 mL of sample three times followed by the measurement of 1 mL of sample in triplicate. The instrument

determines the number of particles per mL that are equal to or greater than 10 μm and the number of particles per mL that are equal to or greater than 25 μm.

The first step in the development of a formulation for the anti-IgE antibody was to determine a suitable buffer and pH for lyophilization and storage of the product. Antibody at a concentration of 5.0 mg/mL was formulated into 10 mM succinate buffers ranging from pH 5.0 to pH 6.5 and into sodium phosphate, potassium phosphate and histidine buffers at pH 7.0. FIG. **9** shows increased antibody aggregate was observed in the higher pH formulations both before and after lyophilization. An exception was the histidine formulation at pH 7, where no increase in aggregate was observed upon storage at 2–8° C. FIG. **10** shows rhuMAb E25 lyophilized in 5 mM histidine buffer at both pH 6 and pH 7 and stored for 1 year at 2–8° C., 25° C., and 40° C. At each assay time point and storage temperature the pH 6 formulation had less aggregate than the antibody formulated at pH 7. These results show histidine at pH 6 is a particularly useful buffer system for preventing aggregation of the antibody.

To facilitate screening of lyoprotectants, the anti-IgE antibody was formulated into sodium succinate at pH 5 with or without a lyoprotectant. Potential lyoprotectants, added at isotonic concentrations, were grouped into 3 categories:

(a) non-reducing monosaccharide (i.e. mannitol);

(b) reducing disaccharides (i.e. lactose and maltose); and

(c) non-reducing disaccharides (i.e. trehalose and sucrose).

Aggregation of the formulations following storage at 2–8° C. and 40° C. for one year is shown in FIGS. **11** and **12**. With storage at 2–8° C., the monosaccharide formulation (mannitol) aggregated at a rate similar to the buffer control, while formulations containing the disaccharides were equally effective in controlling aggregation (FIG. **11**). The results following storage at 40° C. where similar with the exception of the sucrose formulation which rapidly aggregated (which correlated with a browning of the freeze-dried cake (FIG. **12**)). This was later shown to be caused by degradation of sucrose following storage at both acidic pH and high temperature.

Hydrophobic interaction chromatography of the antibody formulated in histidine buffer at pH 6 with lactose shows the antibody is altered following storage for 6 months at 40° C. (FIG. **13**). The chromatography peaks are broadened and the retention time decreases. These changes are not observed with the buffer control and sucrose formulations stored under similar conditions as shown in FIGS. **14** and **15**, respectively. Furthermore, isoelectric focusing showed an acidic shift in the pI of the antibody formulated in lactose and stored at 25° C. and 40° C. This indicates that reducing sugars are not suitable as lyoprotectants for the antibody.

Aggregation of lyophilized formulations of anti-IgE at a concentration of 20 mg/mL in 5 mM histidine buffer at pH 6 with various concentrations of sucrose and trehalose following storage for 12 weeks at 50° C. is shown in FIG. **16**. Both sugars have a similar protective effect on aggregation when the sugar concentration is greater than 500 moles of sugar per mole of antibody. From these results, isotonic and hypertonic formulations of both sucrose and trehalose were identified for further development. The formulations are designed to be filled prior to lyophilization at a relatively low concentration of antibody and the lyophilized product is reconstituted with less volume than was filled with bacteriostatic water for injection (BWFI) comprising 0.9% benzyl alcohol. This allows the concentration of the antibody immediately prior to subcutaneous delivery and includes a preservative for a potential multi-use formulation while avoiding interactions between the protein and preservative upon long-term storage.

Isotonic formulation:

Anti-IgE at 25 mg/mL formulated in 5 mM histidine buffer at pH 6 with 500 moles of sugar per mole antibody which equals a sugar concentration of 85 mM. This formulation is reconstituted with BWFI (0.9% benzyl alcohol) at a volume which is four times less than was filled. This results in a 100 mglmL of antibody in 20 mM histidine at pH 6 with an isotonic sugar concentration of 340 mM.

Hypertonic formulation:

Anti-IgE at 25 mg/mL formulated in 5 mM histidine buffer at pH 6 with 1000 moles of sugar per mole antibody which equals a sugar concentration of 161 mM. This formulation is reconstituted with BWFI (0.9% benzyl alcohol) at a volume which is four times less than was filled. This results in a 100 mg/mL of antibody in 20 mM histidine at pH 6 with a hypertonic sugar concentration of 644 mM.

Comparisons of the antibody aggregation following storage of the isotonic and hypertonic formulations for up to 36 weeks are shown in FIGS. **17** to **19**. No change in aggregation is observed in either the hypertonic or isotonic formulations with storage at 2–8° C. (FIG. **17**). With storage at controlled room temperature (30° C.) increased aggregation is not observed in the hypertonic formulations while an increase in aggregate of from 1 to 2% occurs in the isotonic formulations (FIG. **18**). Finally, following storage at 50° C. a minimal increase in aggregate is observed with the hypertonic formulations, a 4% increase in aggregate occurs with the isotonic trehalose formulation and a 12% increase in aggregate occurs with the isotonic sucrose formulation (FIG. **19**). These results show the isotonic formulation contains the minimum amount of sugar necessary to maintain the stability of the antibody with storage at a temperature up to 30° C.

The binding activity of the anti-IgE in the isotonic and hypertonic formulations was measured in an IgE receptor inhibition assay. It was discovered that the binding activity of the isotonic and hypertonic sucrose and trehalose formulations was essentially unchanged following storage at −70° C., 2–8° C., 300 C and 50° C. for up to 36 weeks.

Lyophilized formulations of proteins are known to contain insoluble aggregates or particulates (Cleland et al., *Critical Reviews in Therapeutic Drug Carrier Systems,* 10 (4):307–377 (1993)). Accordingly, a particulate assay of antibody lyophilized at a concentration of 25 mg/mL in 5 mM histidine, pH 6 with the addition of 85 mM and 161 mM sucrose and trehalose was performed. Polysorbate 20 was added to the formulations at a concentration of 0.005%, 0.01%, and 0.02%. Samples were lyophilized and assayed following reconstitution to 100 mg/mL antibody in 20 mM histidine, pH 6 with 340 mM and 644 mM sugar. The polysorbate 20 concentration following reconstitution was 0.02%, 0.04%, and 0.08%.

Table 9 below shows the number of particles of size equal to or greater than 10 tm and equal to or greater than 25 μm from the isotonic and hypertonic sucrose and trehalose formulations. Polysorbate 20 was added to the formulations at concentrations of 0.005%, 0.01%, and 0.02% prior to lyophilization. The results show that the addition of Tween™ to the formulation significantly reduces the number of particles in each size range tested. The US Pharmacopeia (USP) specification for small volume injections are not more than 6,000 particles of greater than or equal to 10 μm and not more than 600 particles of greater than or equal to 25 μm per container (Cleland et al., supra). With the addition of polysorbate 20, both the hypertonic and isotonic formulations pass this specification.

TABLE 9

Formulation	Polysorbate 20	Particles per mL ≧10 μm	Particles per mL ≧25 μM
Isotonic Sucrose	None	16,122	28
	0.005%	173	2
	0.01%	224	5
	0.02%	303	6
Hypertonic Sucrose	None	14,220	84
	0.005%	73	6
	0.01%	51	0
	0.02%		6
Isotonic Trehalose	None	33,407	24
	0.005%	569	4
	0.01%	991	16
	0.02%	605	9
Hypertonic Trehalose	None	24,967	28
	0.005%	310	11
	0.01%	209	6
	0.02%	344	6

One formulation developed for the anti-IgE antibody (i.e. 143 mg vial isotonic formulation of rhuMAb E25) which is considered to be useful for subcutaneous delivery of this antibody is shown in Table 10 below. A 10 cc vial is filled with 5.7 mL of rhuMAb E25 at a concentration of 25 mg/mL formulated in 5 mM histidine at pH 6.0 with 0.01% polysorbate 20. Sucrose is added as a lyoprotectant at a concentration of 85 mM which corresponds to a molar ratio of sugar to antibody of 500 to 1. The vial is lyophilized and reconstituted with 0.9% benzyl alcohol to one quarter of the volume of the fill or 1.2 mL. The final concentration of components in the formulation is increased four fold to 100 mg/mL rhuMAb E25 in 20 mM histidine at pH 6 with 0.04% polysorbate 20 and 340 mM sucrose (isotonic) and 0.9% benzyl alcohol. The formulation contains histidine buffer at pH 6 because of its demonstrated protective effect on antibody aggregation. Sucrose was added as the lyoprotectant because of previous use in the pharmaceutical industry. The concentration of sugar was chosen to result in an isotonic formulation upon reconstitution. Finally, polysorbate 20 is added to prevent the formation of insoluble aggregates.

TABLE 10

Pre-lyophilized Formulation (Fill 5.7 mL into 10 cc vial)	Reconstituted Formulation (1.2 mL 0.9% Benzyl Alcohol)
25 mg/mL rhuMAb E25	100 mg/mL rhuMAb E25
5 mM Histidine, pH 6.0	20 mM Histidine, pH 6.0
85 mM Sucrose	340 mM Sucrose
0.01% Polysorbate 20	0.04% Polysorbate 20
—	0.9% Benzyl Alcohol

What is claimed is:

1. A stable isotonic reconstituted formulation comprising an antibody in an amount of about 50 mg/mL to about 400 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of the antibody and a lyoprotectant which prevents or reduces chemical or physical instability of the antibody upon lyophilization and subsequent storage, wherein the molar ratio of lyoprotectant:antibody is about 100–510 mole lyoprotectant:1 mole of antibody, and wherein the antibody concentration in the reconstituted formulation is about 2–40 times greater than the antibody concentration in the mixture before lyophilization.

2. The formulation of claim **1** wherein the lyoprotectant is sucrose.

3. The formulation of claim **1** wherein the lyoprotectant is trehalose.

4. The formulation of claim **1** which further comprises a buffer.

5. The formulation of claim **4** wherein the buffer is histidine or succinate.

6. The formulation of claim **1** which farther comprises a surfactant.

7. The formulation of claim **1** which is sterile.

8. A stable reconstituted formulation comprising an antibody in an amount of about 50 mg/mL to about 400 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of an antibody and a lyoprotectant which prevents or reduces chemical or physical instability of the antibody upon lyophilization and subsequent storage, wherein the molar ratio of lyoprotectant:antibody is about 100–510 mole lyoprotectant:1 mole of antibody, and wherein the antibody concentration in the reconstituted formulation is about 2–40 times greater than the antibody concentration in the mixture before lyophilization.

9. The formulation of claim **8** wherein the antibody is an anti-IgE antibody.

10. The formulation of claim **8** wherein the antibody is an anti-HER2 antibody.

11. The formulation of claim **8** wherein the antibody is a full length humanized antibody.

12. The formulation of claim **8** which is isotonic.

13. A method for preparing a stable isotonic reconstituted formulation comprising reconstituting a lyophilized mixture of an antibody and a lyoprotectant which prevents or reduces chemical or physical instability of the antibody upon lyophilization and subsequent storage, wherein the molar ratio of lyoprotectant:antibody is about 100–510 mole lyoprotectant:1 mole of antibody in a diluent such that the antibody concentration in the reconstituted formulation is at least 50 mg/mL, wherein the antibody concentration in the reconstituted formulation is about 2–40 times greater than the antibody concentration in the mixture before lyophilization.

14. The method of claim **13** wherein the lyoprotectant is sucrose.

15. The method of claim **13** wherein the lyoprotectant is trehalose.

16. The method of claim **13** wherein the lyophilized mixture further comprises a bulking agent which is added in an amount which adds mass to the lyophilized mixture and contributes to the physical structure of the lyophilized cake.

17. A method for preparing a formulation comprising the steps of:

(a) lyophilizing a mixture of an antibody and a lyoprotecting amount of a lyoprotectant which prevents or reduces chemical or physical instability of the antibody upon lyophilization and subsequent storage, wherein the molar ratio of lyoprotectant:antibody is about 100–510 mole lyoprotectant:1 mole of antibody; and

(b) reconstituting the lyophilized mixture of step (a) in a diluent such that the reconstituted formulation is isotonic and stable and has an antibody concentration of about 80 mg/mL to about 400 mg/mL.

18. The method of claim **17** wherein the protein concentration in the reconstituted formulation is from about 80 mg/mL to about 300 mg/mL.

19. The method of claim **17** wherein the protein concentration in the reconstituted formulation is about 2–40 times greater than the protein concentration in the mixture before lyophilization.

20. The method of claim **17** wherein lyophilization is performed at a shelf temperature maintained at about 15–30° C. throughout the entire lyophilization process.

21. An article of manufacture comprising:

(a) a container which holds a lyophilized mixture of an antibody and a lyoprotectant which prevents or reduces

chemical or physical instability of the antibody upon lyophilization and subsequent storage, wherein the molar ratio of lyoprotectant: antibody is about 100–510 mole lyoprotectant:1 mole of antibody; and

(b) instructions for reconstituting the lyophilized mixture with a diluent to an antibody concentration in the reconstituted formulation of about 80 mg/mL to about 400 mg/mL.

22. The article of manufacture of claim **21** wherein the protein concentration in the reconstituted formulation is about 2–40 times greater than the protein concentration in the mixture before lyophilization.

23. The article of manufacture of claim **21** further comprising a second container which holds a diluent.

24. The article of manufacture of claim **23** wherein the diluent is bacteriostatic water for injection (BWFI) comprising an aromatic alcohol.

25. A formulation comprising a lyophilized mixture of an antibody and a lyoprotectant which prevents or reduces chemical or physical instability of the antibody upon lyophilization and subsequent storage, wherein the molar ratio of lyoprotectant:antibody is about 100–510 mole lyoprotectant:1 mole antibody.

26. A formulation comprising anti-HER2 antibody in amount from about 5–40 mg/mL, sucrose or trehalose in an amount from about 10–100 mM, a buffer and a surfactant.

27. The formulation of claim **26** further comprising a bulking agent which adds mass to the lyophilized mixture and contributes to the physical structure of the lyophilized cake.

28. The formulation of claim **27** wherein the bulking agent is mannitol or glycine.

29. The formulation of claim **26** which is lyophilized and stable at 30° C. for at least 6 months.

30. The formulation of claim **29** which is reconstituted with a diluent such that the anti-HER2 antibody concentration in the reconstituted formulation is from about 10–30 mg/mL, wherein the reconstituted formulation is stable at 2–8° C. for at least about 30 days.

31. The formulation of claim **30** wherein the diluent is bacteriostatic water for injection (BWFI) comprising an aromatic alcohol.

32. A formulation comprising anti-IgE antibody in amount from about 5–40 mg/ML, sucrose or trehalose in an amount from about 80–300 mM, a buffer and a surfactant.

33. The formulation of claim **32** which is lyophilized and stable at about 30° C. for at least 1 year.

34. The formulation of claim **1** wherein the antibody in the formulation is a monoclonal antibody.

35. The formulation of claim **1** wherein the reconstituted formulation fails to display significant or unacceptable levels of chemical or physical instability of the antibody upon storage at 2–8° C. for at least 30 days.

36. The formulation of claim **1** wherein less than about 10% of the antibody is present as an aggregate in the reconstituted formulation.

37. The formulation of claim **1** wherein less than about 5% of the antibody is present as an aggregate in the reconstituted formulation.

38. The formulation of claim **1** wherein the lyoprotectant is a non-reducing sugar.

39. The formulation of claim **1** wherein the lyoprotectant is a disaccharide.

40. The formulation of claim **39** wherein the lyoprotectant is a non-reducing disaccharide.

41. The formulation of claim **1** wherein the molar ratio of lyoprotectant:antibody is about 200–510 mole lyoprotectant:1 mole of antibody.

42. The article of manufacture of claim **23** wherein the diluent is sterile water.

43. The article of manufacture of claim **23** wherein the diluent is sterile saline solution.

44. The formulation of claim **25** wherein the antibody is a monoclonal antibody.

45. The formulation of claim **44** wherein the lyoprotectant is sucrose.

46. The formulation of claim **44** wherein the lyoprotectant is trehalose.

47. A stable reconstituted formulation comprising a monoclonal antibody in an amount of about 50 mg/mL to about 400 mg/mL and a diluent, which reconstituted formulation has been prepared from a lyophilized mixture of the monoclonal antibody and a sugar selected from the group consisting of sucrose and trehalose, wherein the molar ratio of sugar:monoclonal antibody is about 100–510 mole sugar:1 mole of monoclonal antibody, and wherein the monoclonal antibody concentration in the reconstituted formulation is about 2–40 times greater than the monoclonal antibody concentration in the mixture before lyophilization.

* * * * *

Copia

135

Señora Jefe,

DIVISIÓN DE NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D.

Referencia	**: Expediente N° 07 009315**
Asunto	**: Oposición a la solicitud de patente de Invención titulada "FORMULACIONES PARA ALMACENAR PROTEÍNAS."**
Solicitante	**: WYETH**
Opositora	**:PROCAPS S.A.**

Publicada en la Gaceta de la Propiedad Industrial
N° 581 del 31 de octubre de 2007

Yo, ***ELIANA ISAURA MORENO BOHORQUEZ,*** abogada en ejercicio, identificada como aparece al pie de su firma, con tarjeta profesional No. 83823 del Consejo Superior de la Judicatura, en mi condición de apoderada de la sociedad ***PROCAPS S.A.*** conformada bajo las leyes de la República de Colombia y domiciliada en la ciudad de Barranquilla, Colombia, atentamente manifiesto que estando dentro del término de ley y por orden expresa de mi representada, presento oposición a la concesión del registro de la Patente de Invención titulada **"FORMULACIONES PARA ALMACENAR PROTEÍNAS"**, cuyo titular es **WYETH,** y pido a Usted declarar fundada esta oposición y en consecuencia negar el registro de la patente solicitada.

Carrera 13 N° 119-95, Of. 104 Tel. (571) 2 13 12 13 - 6 20 50 21 - Fax. (571) 6 12 19 79
e-mail: negocios@opumuin-co.com - www.opumuin-co.com
Bogotá Colombia

1. INTERÉS PARA ACTUAR

Conforme a lo dispuesto en el artículo 42 de la Decisión 486 de 2000 de la CAN, estando dentro del término estipulado por la ley, manifiesto el legitimo interés de nuestra representada para oponerse al registro de la patente de la referencia, atendiendo a que la solicitud pretendida no cumple con los requisitos dispuestos en la ley como se analizará más adelante y de ser concedida afectaría directamente la comercialización de algunos productos de mi representada, que contienen, en particular formulaciones de proteínas. Por lo tanto, solicito respetuosamente a ese Despacho se sirva tener en cuenta la presente oposición dentro del trámite administrativo que se surte en esta Superintendencia.

2. FUNDAMENTOS DE HECHOS

2.1. La solicitud objeto de la presente fue presentada el 31 de enero de 2007, reivindicando prioridad bajo las solicitudes US20040601311P del 2004-08-13 y fue publicada en Colombia, en la Gaceta de la Propiedad Industrial N° 581 del 31 de octubre de 2007 página 467 y N° publicación 599, cuya publicación internacional corresponde a la WO2006020935 del 23/02/2006.

2.2. La solicitud colombiana **07-009315** objeto de la presente oposición, se refiere a una formulación, caracterizada porque comprende una proteína aislada; y una solución acuosa que tiene pH desde 4,0 hasta un pH de 8, en donde la formulación no contiene un crioprotector o surfactante, y la proteína es estable por al menos 3 semanas desde una temperatura de -80°C hasta 8°C; donde la proteína es un anticuerpo y la solución acuosa es agua estéril. De igual forma, la solicitud se refiere a un método para su almacenaje; un método para determinar la formulación favorable; y, un polipéptido producido y su uso en la manufactura de proteínas modificadas.

2.3.En primera instancia, se debe advertir que las reivindicaciones que se refieren al método de almacenaje no conforman unidad de invención pues nada tiene que ver una formulación farmacéutica con el método de almacenamiento. Son materias distintas y por lo tanto no conforman unidad de invención. Según la legislación, Decisión 486 de la Comunidad Andina de Naciones, Artículo 36.- "(...) *la oficina nacional competente podrá, en cualquier momento del trámite, requerir al solicitante que divida la solicitud si ella no cumpliera con el requisito de unidad de invención*".

2.4.Ahora bien, con base en el artículo 14 de la Decisión 486 de la CAN consagra lo siguiente: *"Los países miembros otorgarán patentes para las invenciones, sean de producto o procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial"*. Así las cosas, las invenciones objeto de patente deben ser nuevas y tener nivel inventivo. Bien, pues antes de la fecha de prioridad de esta solicitud se encuentran los siguientes documentos que afectan la patentabilidad de la presente solicitud:

- D1: WO9856418 publicada el 17 de diciembre de 1998.
- D2: US6267958 publicada el 31 de julio de 2001.

2.5.Ahora bien, el documento D1 particularmente en las reivindicaciones 1 a 22, páginas 4 a 10, enseña formulaciones acuosas de anticuerpos que comprende de 10 a 25 mM de acetato de sodio con un pH 6 en una concentración de 40 mg/ml. Dicho documento anterior D1 igualmente se refiere a anticuerpos monoclonales, humanizados, anticuerpos recombinantes, disponibles para propósitos terapéuticos, donde dicha formulación revelada es estable de 2 a 8 °C por al menos 2 años (página 6 de D1). De igual forma, esta anterioridad revela que estos anticuerpos son específicos como antígenos sanguíneos tal como Lewis Y o como receptores Erb tal como CD22. De igual forma, esta anterioridad revela que la

formulación de anticuerpos comprende NaCl y un método para estabilizar dicho anticuerpo en la página 24 a 46. Así, las cosas la presente solicitud no es novedosa pues lo revelado ya se encontraba en el estado de la técnica, particularmente en el documento D1.

2.6. El documento D2, según la descripción y particularmente la cláusulas 1 a 47 revela por su parte formulaciones acuosas de anticuerpos que comprenden de 10 a 25 mM de acetato de sodio con un pH 6 en una concentración de 30 mg/ml. Según dicho documento anterior D2 particularmente en la columna 6 a 13, también se refiere a anticuerpos monoclonales, humanizados, anticuerpos recombinantes, disponibles para propósitos terapéuticos, donde dicha formulación revelada es estable de 2 a 8 °C por al menos 2 años. De igual forma, esta anterioridad D2 anticipa que estos anticuerpos son específicos como antígenos sanguíneos (tal como Lewis Y) o como receptores Erb (tal como CD22) y agrega en la columna 14 a 24 que la formulación de anticuerpos comprende NaCl y un método para estabilizar dicho anticuerpo. Por lo tanto, este documento objeto de oposición a la luz del documento D2 carece evidentemente de NOVEDAD.

2.7. En consecuencia y de acuerdo a lo descrito anteriormente, se deriva que la solicitud colombiana **07 009315** no puede pretender un monopolio por patente toda vez que lo que pretende no cumple con uno de los tres requisitos necesarios para su protección.

2.8. Que por consiguiente, respecto a la solicitud pretendida se tiene que:

2.8.1. **CARECE DE NOVEDAD**: Según la decisión 486 de la Comunidad Andina de Naciones, Artículo 16.- *"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."*. A la fecha de prioridad, 13 de agosto de 2004 ya era

conocido, pues como se demostró, era parte del arte anterior con base en los documentos D1 y D2, una formulación que comprende una proteína aislada; y una solución acuosa que tiene pH desde 4,0 hasta un pH de 8, en donde la formulación no contiene un crioprotector o surfactante, y la proteína es estable por al menos 3 semanas desde una temperatura de -80°C hasta 8°C, sobre el cual busca protección en el capítulo reivindicatorio.

2.9. Dado lo anterior, es posible deducir que la solicitud y lo reivindicado no es objeto de patentabilidad pues es contrario a lo que dicta la Decisión 486 de la Comunidad Andina de Naciones, Artículo 14.- *"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."*. La solicitud pretendida no cumple con el requisito de NOVEDAD, uno de los tres requisitos necesarios para su patentabilidad.

2.10. Por todo lo anterior, atentamente solicito a usted que se sirva declarar fundada la presente oposición y negar el registro Patente de invención denominada **"FORMULACIONES PARA ALMACENAR PROTEÍNAS"**, cuyo titular es **WYETH,,** solicitud Colombiana que apareció publicada en la Gaceta de la Propiedad Industrial N° 581.

3. PRUEBAS

De conformidad con lo dispuesto por las normas vigentes, solicito a usted tener como prueba en este asunto los siguientes:

- D1: WO9856418 publicada el 17 de diciembre de 1998.

- D2: US6267958 publicada el 31 de julio de 2001.

4. FUNDAMENTO DE DERECHO

Fundamento esta observación en las normas legales siguientes:

4.1. Numeral 1 del artículo 586 del Código de Comercio.

4.2. Artículo 42 de la Decisión 486 de la Comisión del Acuerdo de Cartagena.

4.3. En las demás normas concordantes.

5. ANEXOS

Para los efectos correspondientes anexo al presente escrito los siguientes documentos:

1. Poder para actuar en el presente.
2. Documento que acredita al representante legal de PROCAPS S.A.
3. Recibo de caja del respectivo pago de tasa.
4. Copias de los documentos:

- D1: WO9856418 publicada el 17 de diciembre de 1998.
- D2: US6267958 publicada el 31 de julio de 2001.

6. NOTIFICACIONES

Para efectos de notificaciones que por disposición del artículo 50 del Decreto 01 de 1984 debe hacer su Despacho, informo que mi representada y la suscrita recibiremos notificaciones en la Secretaría de su Despacho o en mis oficinas situadas en la Carrera 13 N° 119-95, Of. 104 de esta ciudad de Bogotá.

Señor Superintendente,

ELIANA ISAURA MORENO BOHORQUEZ
C.C. 51'975.664 de Bogotá
T.P.A. 83823 del C.S. de la J.