SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RAD: 15-137634- -1 **FECHA**: 2015-08-26 09:54:24
DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 10
ACT: 476 TRASTITULARSOLI

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
MARGARITA CASTELLANOS ABONDANO

Asunto: Radicación: 15-137634- -1
Trámite: 11
Evento: 378
Actuación: 476
Oficio: 10144
Folios: 10

Patente de Invención	**X**			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	**X**	Capítulo I	**X**	Capítulo II	
No. Solicitud Internacional	PCT/US2012/037394				
Fecha de Presentación Internacional	10/05/2012				

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

1. Documentos estudiados

Descripción:	Radicado N° 15-137634-00000-0000	16/junio/2015
Reivindicaciones:	Radicado N° 15-137634-00000-0000	16/junio/2015
Secuencias:	Radicado N° 15-137634-00000-0000	16/junio/2015
Dibujos:	Radicado N° 15-137634-00000-0000	16/junio/2015
Prioridad:	US 61 484610	10/Mayo/2011
	US 61 562303	21/Noviembre/2011
	US 61 595526	06/Febrero/2012
	US 61 614417	22/Marzo/2012
	US 61 642363	03/Mayo/2012

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Presentación de solicitudes Fraccionarias (Art 36 D 486)

Se acepta para examen la solicitud fraccionaria presentada e identificada con el radicado N°15-137634-00000-0000, porque no implican ampliación de la materia contenida en la solicitud inicial con radicado N°13-286712-00000-0000.

4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capitulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por la solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiarán las reivindicaciones 1 a 34 del capítulo reivindicatorio (Radicado N°. 15-137634-00000-0000).

Título de la solicitud: “MÉTODOS PARA TRATAR O PREVENIR TRANSTORNOS RELACIONADOS CON EL COLESTEROL”

El problema planteado en esta solicitud consiste en la necesidad de obtener nuevos métodos para tratar o prevenir trastornos relacionados con el colesterol, formulaciones y los métodos para producir dichas formulaciones.

Para resolver este problema técnico la solicitud en estudio proporciona métodos para tratar o prevenir trastornos relacionados con el colesterol, formulaciones y los métodos para producir dichas formulaciones.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 16/40, A61K 39/395, A61P 3/06

5. De la solicitud de patente

Titulo

El título hace referencia a métodos para tratar o prevenir trastornos; los métodos de tratamientos son considerados una excepción a la patentabilidad, por lo cual se sugiere a la solicitante hacer las aclaraciones pertinentes.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es clara puesto que hace referencia a términos “aproximadamente”, “al menos”, “farmacéuticamente aceptable”, los cuales son imprecisos y no permiten distinguir la invención del estado de la técnica, además menciona el enlace del anticuerpo que se une específicamente a PCSK9, lo cual es considerado un resultado a alcanzar y no define el anticuerpo por sus características esenciales, puesto que la reivindicación incluiría no sólo la solución propuesta por la solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se le recuerda a la solicitante que un anticuerpo debe ser caracterizado a partir de su estructura completa es decir, las secuencias que lo conforman en la cadena ligera y cadena pesada, con su respectivo SEQ ID NO, las secuencias variables de la cadena ligera y pesada o las secuencias de los 6 CDR´s que conforman ambas cadenas. Además la reivindicación 1 no es clara porque no define una formulación en términos de los ingredientes, excipientes en las cantidades o proporciones específicas que la conforman.

Las reivindicaciones 2, 29, 30, 33 y 34 no son claras, ni tienen sustento porque mencionan la expresión “al menos el 90% idéntica”, lo cual no tiene sustento en la descripción ya que hace referencia a porcentajes de identidad de las secuencias reclamadas. Se sugiere al solicitante caracterizar las posibles variantes estructurales de las secuencias citadas en estas reivindicaciones por sus secuencias completas, las cuales además deben ser adjuntadas en un listado aparte con su respectivo SEQ ID NO, dichas variantes deben estar completamente sustentadas en la descripción. Por último se recuerda a la solicitante que no se aceptan definiciones indicando porcentajes de identidad, puesto que esta definición no es clara, por lo tanto no permite establecer la secuencia de aminoácidos exacta a la que hace referencia la solicitud.

La reivindicación 4 no es clara puesto que menciona los anticuerpos monoclonales por medio de nombres comercial farmacéutico, lo que no permite distinguir la invención del estado de la técnica, además no es concisa puesto que menciona una formulación la cual comprende un anticuerpo de la selección en 11F1, 31H4, 8A3, 8A1, lo cual tampoco permite distinguir el objeto concreto de dicha reivindicación. Se sugiere caracterizar el anticuerpo estructuralmente mencionando las secuencias de los 3 CDRs de la cadena pesada y los 3 CDRs de la cadena ligera, así mismo las secuencias de cadena pesada y ligera. Las reivindicaciones deben ser claras por si solas sin referencia a nombres comunes.

La reivindicación 5 no es concisa puesto que hace referencia a la selección de un tampón farmacéuticamente aceptable en un grupo que consiste en glutamato, fosfato, salina con tampón de fosfato, acetato sódico, citrato de sodio y tampón tris, lo cual no permite distinguir el objeto concreto de dicha reivindicación.

Las reivindicaciones 11, 17, 19-21, 24-26, 30, 32 y 34 no son claras puesto que mencionan el término “aproximadamente”, el cual es impreciso, por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término rango concreto.

La reivindicación 12 no es concisa puesto que hace referencia a la selección un estabilizador farmacéuticamente aceptable seleccionado del grupo que consiste en polihidroxi hidrocarburo, un disacárido, un poliol, prolina, arginina, lisina, metionina, taurina y alcohol bencílico, lo cual no permite distinguir el objeto concreto de dicha reivindicación. Además se evidencia que no hay sustento por medio de pruebas técnicas para todos los estabilizadores mencionados en dicha reivindicación.

La reivindicación 13 no es concisa puesto que menciona la selección un estabilizador que es polihidroxi hidrocarburo seleccionado del grupo que consiste en sorbitol, manitol y glicerol, lo cual no permite distinguir el objeto concreto de dicha reivindicación. Además se evidencia que no hay sustento por medio de pruebas técnicas.

La reivindicación 15 no es concisa puesto que menciona la selección un estabilizador que es disacárido seleccionado del grupo que consiste en sucrosa, maltosa, lactosa, fructosa y trehalosa, lo cual no permite distinguir el objeto concreto de dicha reivindicación. Además se evidencia que no hay sustento por medio de pruebas técnicas para los estabilizadores maltosa, lactosa, fructosa ni trehalosa.

Las reivindicaciones 27 y 28 mencionan que la formulación permanece estable durante al menos 3, 6, 12 o 24 meses, lo cual es considerado un resultado a alcanzar y no define la formulación por sus características esenciales, puesto que la reivindicación incluiría no sólo la solución propuesta por la solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere caracterizar la formulación por sus características estructurales, componentes activos, cantidades, ingredientes y las proporciones de los mismos.

Las reivindicaciones 33 y 34 no son concisas porque se presenta por categoría inventiva de producto más de una reivindicación independiente. Se le recuerda a la solicitante que por categoría inventiva de producto solo puede haber una reivindicación independiente.

Las reivindicaciones 22 a 26 hacen referencia a una formulación estable caracterizando dicha formulación por la osmolaridad y los valores de viscosidad, lo cual es considerado un resultado a alcanzar y no define la formulación por sus características esenciales, puesto que la reivindicación incluiría no sólo la solución propuesta por la solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere caracterizar la formulación por sus características estructurales, componentes activos, cantidades, ingredientes y las proporciones de los mismos.

Descripción (Art 28 D 486)

La descripción no divulga la invención de manera suficientemente completa para que la persona del oficio normalmente versada en la materia pueda ejecutarla. Se evidencia que el objeto de la solicitud son métodos para el tratamiento y prevención de trastornos relacionados con el colesterol, además de una formulación la cual contiene anticuerpos anti-PCSK9 para el tratamiento de dichos trastornos. Se le recuerda a la solicitante que los métodos de tratamiento son considerados una excepción a la patentabilidad.

Se requiere a la solicitante hacer las aclaraciones necesarias.

6. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 6, grupos inventivos:

Los grupos inventivos están definidos por las siguientes características esenciales:

Anticuerpo	Ligera/pesada SEC ID N°:
31H4	12/67
11F1	465/463
8 A3	461/459
8 A1	485/483
Consenso CDRs 11F1, 8 A3,8 A1	582/583
no tiene sustento	577/576
no tiene sustento	588/589
no tiene sustento	588/599

1. Una formulación estable que comprende un anticuerpo monoclonal con secuencia de aminoácidos de cadena ligera SEQ ID N°. 461, 465,485 o 582 y una secuencia de aminoácidos de cadena pesada SEQ ID N°: 459, 463, 483 o 583 respectivamente, en una cantidad de entre aproximadamente 40mg/ml y aproximadamente 300mg/ml, un tampón farmacéuticamente aceptable en una cantidad de entre aproximadamente 0,05Mm y aproximadamente 40Mm, un surfactante farmacéuticamente aceptable en una cantidad que es entre aproximadamente el 0,01% p/v y aproximadamente el 20% p/v, al menos un estabilizador farmacéuticamente aceptable de entre aproximadamente el 0,5% p/v y aproximadamente el 10% p/v en donde la formulación estable tiene un ph de entre aproximadamente 4,0 y aproximadamente 6,0. (reivindicaciones 1-32)
2. Una formulación estable que comprende un anticuerpo monoclonal con secuencia de aminoácidos de cadena ligera SEQ ID N°. 12 y una secuencia de aminoácidos de cadena pesada SEQ ID N°: 67, en una cantidad de entre aproximadamente 40mg/ml y aproximadamente 300mg/ml, un tampón farmacéuticamente aceptable en una cantidad de entre aproximadamente

0,05Mm y aproximadamente 40Mm, un surfactante farmacéuticamente aceptable en una cantidad que es entre aproximadamente 0,01% p/v y aproximadamente 20% p/v, al menos un estabilizador farmacéuticamente aceptable de entre aproximadamente 0,5% p/v y aproximadamente 10% p/v en donde la formulación estable tiene un ph de entre aproximadamente 4,0 y aproximadamente 6,0. (reivindicaciones 1-36)

3. Una formulación estable que comprende un anticuerpo monoclonal con secuencia de aminoácidos de cadena ligera SEQ ID N°. 577 y una secuencia de aminoácidos de cadena pesada SEQ ID N°: 576, en una cantidad de entre aproximadamente 70mg/ml y aproximadamente 200mg/ml, 10 Mm de acetato de sodio; 9,0 % p/v de sucrosa; entre aproximadamente 0,0004% y aproximadamente 0,01% p/v de polisorbato 20 o polisorbato 80 y; un ph de aproximadamente 5,2.- (reivindicación 37) - no tiene sustento.
4. Una formulación estable que comprende un anticuerpo monoclonal con secuencia de aminoácidos de cadena ligera SEQ ID N°. 588 y una secuencia de aminoácidos de cadena pesada SEQ ID N°: 599, en una cantidad de entre aproximadamente 70mg/ml y aproximadamente 200mg/ml, 10 Mm de acetato de sodio; 9,0 % p/v de sucrosa; entre aproximadamente 0,0004% y aproximadamente 0,01% p/v de polisorbato 20 o polisorbato 80 y; un ph de aproximadamente 5,2.- (reivindicación 37) - no tiene sustento.
5. Una formulación estable que comprende un anticuerpo monoclonal con secuencia de aminoácidos de cadena ligera SEQ ID N°. 577 y una secuencia de aminoácidos de cadena pesada SEQ ID N°: 576, en una cantidad de entre aproximadamente 70mg/ml y aproximadamente 200mg/ml, 10 Mm de acetato de sodio; aproximadamente 2,0 % p/v y aproximadamente el 3,0% p/v de prolina; 0,01% p/v de polisorbato 20 o polisorbato 80 y; un ph de aproximadamente 5,0. (reivindicación 38) - no tiene sustento.
6. Una formulación estable que comprende un anticuerpo monoclonal con secuencia de aminoácidos de cadena ligera SEQ ID N°. 588 y una secuencia de aminoácidos de cadena pesada SEQ ID N°: 589, en una cantidad de entre aproximadamente 70mg/ml y aproximadamente 200mg/ml, 10 Mm de acetato de sodio; aproximadamente 2,0 % p/v y aproximadamente el 3,0% p/v de prolina; 0,01% p/v de polisorbato 20 o polisorbato 80 y; un ph de aproximadamente 5,0. (reivindicación 38) - no tiene sustento.

Se realizó alineamiento de las regiones variables de las cadenas pesadas y livianas obteniendo los siguientes resultados:

Alineamiento de las regiones variables de la cadena pesada:

```
Percent Identity  Matrix - created by Clustal2.1


  1: seq463      100.00   96.85   92.91   92.13   90.55   78.86   74.58
  2: seq459       96.85  100.00   92.91   92.13   90.55   80.49   73.73
  3: seq576       92.91   92.91  100.00   88.19   86.61   78.05   72.03
  4: seq483       92.13   92.13   88.19  100.00   90.55   78.05   74.58
  5: seq583       90.55   90.55   86.61   90.55  100.00   75.61   72.03
  6: seq67        78.86   80.49   78.05   78.05   75.61  100.00   77.12
  7: seq589       74.58   73.73   72.03   74.58   72.03   77.12  100.00
```

Alineamiento de las regiones variables de la cadena liviana:

```
Percent Identity  Matrix - created by Clustal2.1


  1: seq461      100.00  100.00   98.21   97.32   96.43   70.54   48.62
  2: seq485      100.00  100.00   98.21   97.32   96.43   70.54   48.62
  3: seq577       98.21   98.21  100.00   97.32   97.32   70.54   47.71
  4: seq465       97.32   97.32   97.32  100.00   96.43   68.75   48.62
  5: seq82        96.43   96.43   97.32   96.43  100.00   68.75   47.71
  6: seq588       70.54   70.54   70.54   68.75   68.75  100.00   51.38
  7: seq12        48.62   48.62   47.71   48.62   47.71   51.38  100.00
```

No hay unidad de invención entre los grupos inventivos mencionados anteriormente ya que los anticuerpos mencionados en la solicitud son estructuralmente distintos, y ya es plenamente conocido en el estado de la técnica anticuerpos que interactúan con proteina convertasa subtilisina Kexin Tipo 9 (PCSK9), lo cual es enseñado en el documento D1, además el documento D2 revela el desarrollo de formulaciones proteicas estables empleando anticuerpos monoclonales humanizados. (Análisis de patentabilidad grupo inventivo 1 a 6).

Se le sugiere al solicitante restringir la materia a patentar en un solo concepto común inventivo especificando el avance tecnológico sobre el estado de la técnica, eliminando de ella las otras invenciones o presentar las solicitudes divisionales que se consideren necesarias teniendo en cuenta su respectivo sustento dentro de la memoria descriptiva.

Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 10 de mayo de 2011, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	WO 2009026558	Antigen binding proteins to proprotein convertase subtilisin kexin type 9 (pcsk9)	26/Febrero/2009
D2	CLELAND J *et al*., 2001. Journal of pharmaceutical sciences. Vol 90, No. 3, pág. 310 – 321.	A Specific Molar Ratio of Stabilizer to Protein is Required for Storage Stability of a Lyophilized Monoclonal Antibody	Marzo/2001

El documento D1[1] divulga anticuerpos que interactúan con la proteína convertasa subtilisina Kexin Tipo 9 (PCSK9), además se describen métodos de tratamiento de la hipercolesterolemia y otros trastornos mediante la administración de una cantidad farmacéuticamente eficaz de una proteína de unión al antígeno PCSK9 y métodos de detección de la cantidad de PCSK9 en una muestra (Resumen).

El documento D2[2] divulga un estudio sobre el desarrollo de formulaciones proteicas estables empleando un anticuerpo monoclonal humanizado. Se emplean proporciones molares de azucares no reductores respecto al anticuerpo y se determina la estabilidad de las formulaciones liofilizadas resultantes mediante la medición de la agregación, desamidación y la oxidación de la proteína reconstituida y por espectroscopia IR de la proteína seca (Resumen).

7. Examen de Patentabilidad (Art 14 D486)

Grupo inventivo 1 a 6

1 http://worldwide.espacenet.com/publicationDetails/description?CC=WO&NR=2009026558A1&KC=A1&FT=D&ND=&date=20090226&DB=&&locale=en_EP
2 http://www.ncbi.nlm.nih.gov/pubmed/11170024

Nivel inventivo (Art 18 D486)

La reivindicación 1 consiste en: *"Una formulación estable que comprende al menos un anticuerpo monoclonal que se une específicamente a PCSK9, en donde PCSK9 comprende los aminoácidos de SEQ ID NO: 1, en anticuerpo monoclonal en una cantidad de entre aproximadamente 40mg/ml y entre aproximadamente 300 mg/ml, un tampón farmacéuticamente aceptable en una cantidad de entre aproximadamente 0,05Mm y aproximadamente 40Mm, un surfactante farmacéuticamente aceptable en una cantidad que es entre aproximadamente el 0,01% p/v y aproximadamente el 20% p/v, al menos un estabilizador farmacéuticamente aceptable de entre aproximadamente el 0,5% p/v y aproximadamente el 10% p/v en donde la formulación estable tiene un pH de entre aproximadamente 4,0 y aproximadamente 6,0"* (Radicado Nº. 15-137634-00000-00000).

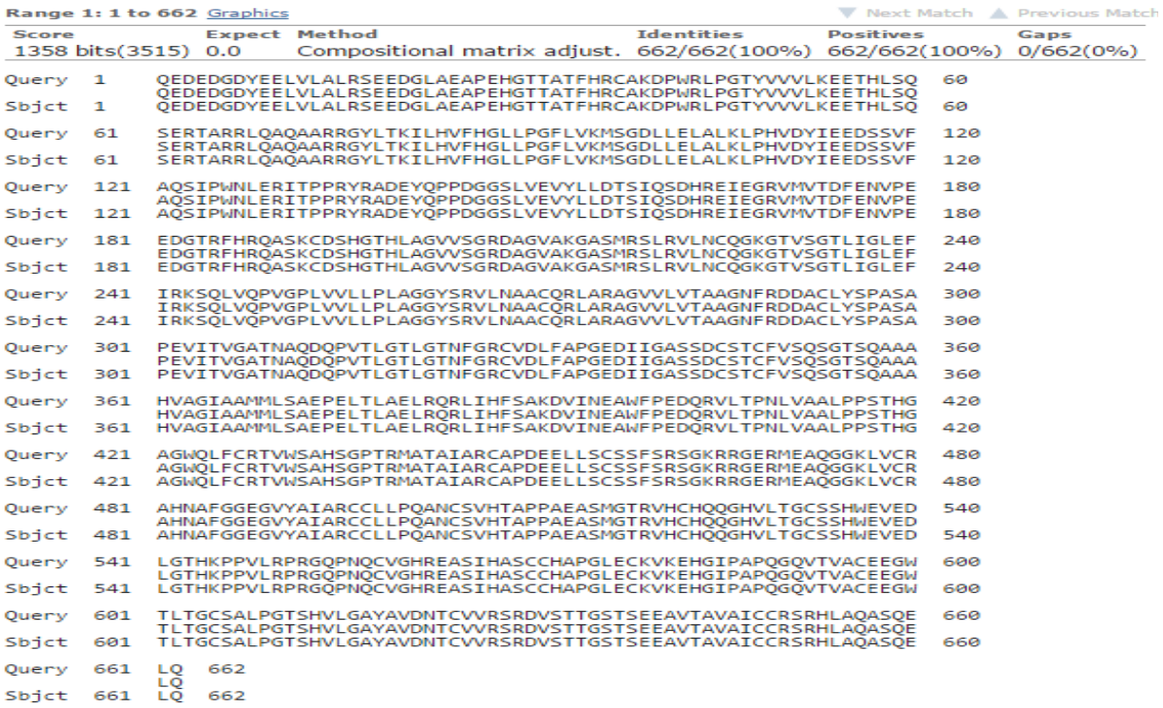
Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Formulación estable que comprende al menos un anticuerpo monoclonal	Formulación que contiene anticuerpos monoclonales "proproteína convertasa subtilisina Kexin tipo 9" o "PCSK9" (Párr. [0244]-[0370]) se refiere a un polipéptido que se expone en la SEQ ID NO: 1 (100% idéntica a la SEQ. 1. N°:1) (Párr. [0165])	Formulación del anticuerpo rhuMAb HER2, que comprende el almacenamiento a temperaturas elevadas, para determinar su estabilidad relativa (Pág. 311, Col. 2, Párr. 1).
Anticuerpo monoclonal en una cantidad de entre aproximadamente 40mg/ml y aproximadamente 300mg/ml	Proteína de unión es un anticuerpo monoclonal, un anticuerpo policlonal, un anticuerpo recombinante, un anticuerpo humano, un anticuerpo humanizado, un anticuerpo quimérico, un anticuerpo multiespecífico, o un fragmento de este anticuerpo. (Párr. [0013])	Anticuerpo monoclonal humanizado (rhuMAb HER2, La proteína se formuló a 25 mg / mL (Pág. 312)
Tampón farmacéuticamente aceptable en una cantidad de entre aproximadamente 0,05Mm y aproximadamente 40Mm	Tampones (tales como borato, bicarbonato, Tris-HCl, citratos, fosfatos u otros ácidos orgánicos) (Párr. [0375])	NaCl gradiente en 20 mM de tampón de fosfato de Na. (Pág. 312)
Surfactante farmacéuticamente aceptable en una cantidad que es entre aproximadamente el 0,01% p/v y aproximadamente el 20% p/v	Agentes de suspensión; tensioactivos o agentes humectantes, polisorbato 20, polisorbato 80 (Párr. [0375])	0.01% polysorbate 20 (Pág. 312)
Estabilizador farmacéuticamente aceptable de entre aproximadamente el 0,5% p/v y aproximadamente el 10% p/v	Agentes (tales como sacarosa o sorbitol) mejorar la estabilidad; agentes potenciadores de la tonicidad (tales como haluros de metales alcalinos, preferiblemente sodio o cloruro de potasio, manitol sorbitol) (Párr. [0375])	0 -250 mM sucrosa. (Pág. 313)
Formulación estable tiene un ph de entre aproximadamente 4,0 y aproximadamente 6,0	Composiciones farmacéuticas comprenden tampón Tris de aproximadamente pH 7,0-8,5, o tampón acetato de aproximadamente pH 4,0-5,5. (Párr. [0378])	pH 6 (Pág. 312)

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga una formulación estable que comprende al menos un anticuerpo monoclonal que se une específicamente a PCSK9, en el que PCSK9 (Párr. [0244]-[0370]) comprende los aminoácidos de SEQ ID NO 1 (Párr. [0165]); 100% de identidad con la SEQ ID NO: 1 de la solicitud, además de un tampón aceptable en una cantidad de aproximadamente 0,05 mM a aproximadamente 40 mM, un agente tensioactivo farmacéuticamente aceptable, y al menos un estabilizador

farmacéuticamente aceptable, disacáridos, lisina, sacarosa 9% (Párr. [0375]), en el que la formulación estable tiene un pH de entre aproximadamente 4,0 a aproximadamente 6,0 (Párr. [0376, 0377, 0378, 0445]).

En el alineamiento se evidencia que la SEC ID NO: 1 de la solicitud es 100% idéntica a la SEQ ID NO: 1, del documento D1.



Range 1: 1 to 662 Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
1358 bits(3515)	0.0	Compositional matrix adjust.	662/662(100%)	662/662(100%)	0/662(0%)

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            QEDEDGDYEELVLALRSEEDGLAEAPEHGTTATFHRCAKDPWRLPGTYVVVLKEETHLSQ
Sbjct  1    QEDEDGDYEELVLALRSEEDGLAEAPEHGTTATFHRCAKDPWRLPGTYVVVLKEETHLSQ  60

Query  61   SERTARRLQAQAARRGYLTKILHVFHGLLPGFLVKMSGDLLELALKLPHVDYIEEDSSVF  120
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Sbjct  61   SERTARRLQAQAARRGYLTKILHVFHGLLPGFLVKMSGDLLELALKLPHVDYIEEDSSVF  120

Query  121  AQSIPWNLERITPPRYRADEYQPPDGGSLVEVYLLDTSIQSDHREIEGRVMVTDFENVPE  180
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Query  181  EDGTRFHRQASKCDSHGTHLAGVVSGRDAGVAKGASMRSLRVLNCQGKGTVSGTLIGLEF  240
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Sbjct  181  EDGTRFHRQASKCDSHGTHLAGVVSGRDAGVAKGASMRSLRVLNCQGKGTVSGTLIGLEF  240

Query  241  IRKSQLVQPVGPLVVLLPLAGGYSRVLNAACQRLARAGVVLVTAAGNFRDDACLYSPASA  300
            IRKSQLVQPVGPLVVLLPLAGGYSRVLNAACQRLARAGVVLVTAAGNFRDDACLYSPASA
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Query  301  PEVITVGATNAQDQPVTLGTLGTNFGRCVDLFAPGEDIIGASSDCSTCFVSQSGTSQAAA  360
            PEVITVGATNAQDQPVTLGTLGTNFGRCVDLFAPGEDIIGASSDCSTCFVSQSGTSQAAA
Sbjct  301  PEVITVGATNAQDQPVTLGTLGTNFGRCVDLFAPGEDIIGASSDCSTCFVSQSGTSQAAA  360

Query  361  HVAGIAAMMLSAEPELTLAELRQRLIHFSAKDVINEAWFPEDQRVLTPNLVAALPPSTHG  420
            HVAGIAAMMLSAEPELTLAELRQRLIHFSAKDVINEAWFPEDQRVLTPNLVAALPPSTHG
Sbjct  361  HVAGIAAMMLSAEPELTLAELRQRLIHFSAKDVINEAWFPEDQRVLTPNLVAALPPSTHG  420

Query  421  AGWQLFCRTVWSAHSGPTRMATAIARCAPDEELLSCSSFSRSGKRRGERMEAQGGKLVCR  480
            AGWQLFCRTVWSAHSGPTRMATAIARCAPDEELLSCSSFSRSGKRRGERMEAQGGKLVCR
Sbjct  421  AGWQLFCRTVWSAHSGPTRMATAIARCAPDEELLSCSSFSRSGKRRGERMEAQGGKLVCR  480

Query  481  AHNAFGGEGVYAIARCCLLPQANCSVHTAPPAEASMGTRVHCHQQGHVLTGCSSHWEVED  540
            AHNAFGGEGVYAIARCCLLPQANCSVHTAPPAEASMGTRVHCHQQGHVLTGCSSHWEVED
Sbjct  481  AHNAFGGEGVYAIARCCLLPQANCSVHTAPPAEASMGTRVHCHQQGHVLTGCSSHWEVED  540

Query  541  LGTHKPPVLRPRGQPNQCVGHREASIHASCCHAPGLECKVKEHGIPAPQGQVTVACEEGW  600
            LGTHKPPVLRPRGQPNQCVGHREASIHASCCHAPGLECKVKEHGIPAPQGQVTVACEEGW
Sbjct  541  LGTHKPPVLRPRGQPNQCVGHREASIHASCCHAPGLECKVKEHGIPAPQGQVTVACEEGW  600

Query  601  TLTGCSALPGTSHVLGAYAVDNTCVVRSRDVSTTGSTSEEAVTAVAICCRSRHLAQASQE  660
            TLTGCSALPGTSHVLGAYAVDNTCVVRSRDVSTTGSTSEEAVTAVAICCRSRHLAQASQE
Sbjct  601  TLTGCSALPGTSHVLGAYAVDNTCVVRSRDVSTTGSTSEEAVTAVAICCRSRHLAQASQE  660

Query  661  LQ  662
            LQ
Sbjct  661  LQ  662
```

La diferencia entre la invención, definida en la reivindicación 1 y la formulación divulgada en el documento D1 consiste en que la presente solicitud hace referencia al anticuerpo monoclonal que se encuentra en una cantidad de aproximadamente 40 mg/ml a aproximadamente 300 mg/ml, y el agente tensioactivo aceptable está en una cantidad que es de aproximadamente 0,01% w/v a aproximadamente 20 % w/v.

No se evidencia un efecto técnico asociado en la formulación mencionada en la presente solicitud con relación en la mencionada en el documento D1.

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se puede formular así: “Proporcionar formulaciones alternativas, para tratar o prevenir trastornos relacionados con el colesterol”

Sin embargo, el documento D2 hace referencia a formulaciones farmacéuticamente aceptables con anticuerpo monoclonal humanizado (rhuMAb HER2, la proteína se formuló a 25 mg/mL y sus diferentes componentes (Pág. 312-313) y el ejemplo 13 menciona soluciones que comprenden 2mg/ml de anticuerpo anti-31H4 PCSK9 en PBS a un nivel de 10mg/kg a través de la vena de un ratón. Un ser humano con 70Kg necesitaría forma 7[mu]g/70kg hasta aproximadamente 7000mg/70kg si se extrapola linealmente. Por lo tanto para proporcionar la cantidad extrapolada en una concentración de 40 mg/ml a 300 mg/ml cae dentro de la práctica común de una persona versada en la materia; así mismo las cantidades a emplear de los componentes ya conocidos en el estado de la técnica para generar formulaciones estables.

En consecuencia, la persona del oficio normalmente versada en la materia se vería motivada a proponer nuevas formulaciones estables anti-PCSK9, con el fin de que sean utilizados para el tratamiento de trastornos relacionados con el colesterol mencionados en el documento D2, con los diferentes componentes también mencionados en el

documento D1 para realizar formulaciones estables, para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Puesto que las reivindicaciones dependientes 2-32 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Las reivindicaciones 33 y 34 hacen referencia a una formulación estable que comprende al menos un anticuerpo monoclonal que se une específicamente a PCSK9, no mencionan características inventivas adicionales, por lo tanto tampoco se pueden considerar con nivel inventivo.

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO 2009028938	1-34	Nivel inventivo
D2	CLELAND J *et al*., 2001. Journal of pharmaceutical sciences. Vol 90, No. 3, pág. 310 – 321.	1-34	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2015-09-01

Elaboró: Juanita Andrea Carvajal
Revisó: Sandra Carolina Crespo

INFORME DE BÙSQUEDA DE PATENTES PARA EL No de Radicación: 15-137634-00000-0000	
CRITERIOS PARA LA BÙSQUEDA (anexar pantallazo o reporte en PDF, junto con los boleanos utilizados)	
Palabras clave utilizadas	Methods Of Treating Or Preventing Cholesterol Related Disorders, antibody PCSK9
CLASIFICACIONES INTERNACIONALES UTILIZADAS PARA LA BÚSQUEDA	

BASES DE DATOS CONSULTADAS.			
NCBI	The Lens		
Google/patents	ClustalW		
Patentscope			

INFORMES DE EXÁMENES REALIZADOS EN OTRAS OFICINAS (documentos patentes, literatura no patente etc…)

Oficina	Fecha del examen o de reporte de búsqueda de documentos	Anterioridades encontradas para el examen o reportados en el examen de búsqueda(relacionarlos todos)	Reiv. afectadas	RELEVANCIA (requisito que afecta)	FUERON TENIDOS EN CUENTA PARA EL EXAMEN EN LA OFICINA COLOMBIANA	
					SI	NO
EUROPA	12/Noviembre/2013	WO 2011028938	1-70	Nivel Inventivo		X
		WO 2009026558	1-70	Nivel Inventivo	X	

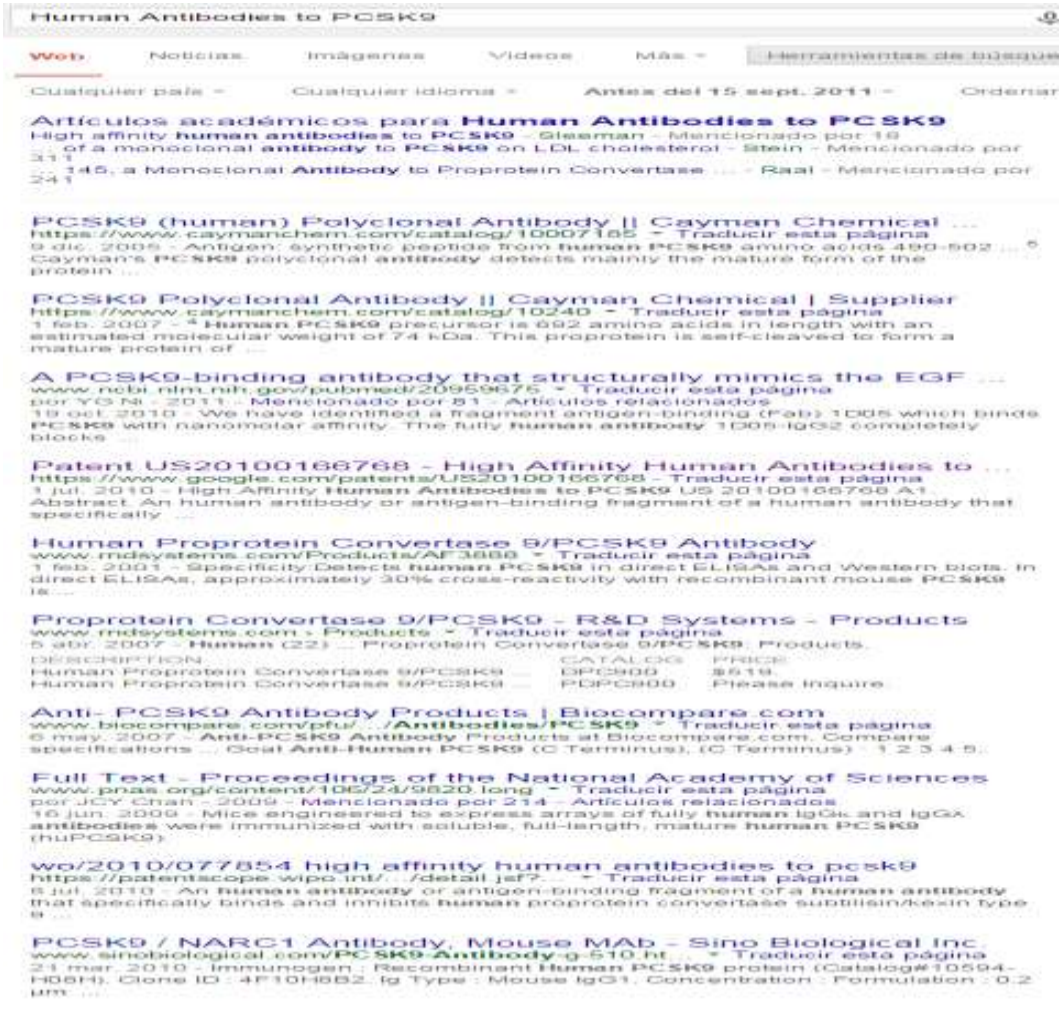
DOCUMENTOS ADICIONALES ENCONTRADOS POR EL EXAMINADOR DE ACUERDO A LOS CRITERIOS DE BÙSQUEDA.

A Specific Molar Ratio of Stabilizer to Protein is Required for Storage Stability of a Lyophilized Monoclonal Antibody

BÚSQUEDA DE SECUENCIAS
Número de depósito / patente y N° de secuencia
% de identidad

SEQ ID NO (de la solicitud)	NCBI	The Lens	EMBL-EBI	DDJB	Genome Quest
SEQ 1	99% idéntica a la secuencia proteína convertasa subtilisin/ kevin tipo 9 *Homo sapiens*				
SEQ 465		100% de identidad a la SEQ ID NO 465 del documento WO 2009026558			
SEQ 12		100% de identidad a la SEQ ID NO 1 del documento WO 2009026558			
SEQ 67		100% de identidad a la SEQ ID NO 67 del documento WO 2009026558			
SEQ 459		100% de identidad a la SEQ ID NO 459 del documento WO 2009026558			
SEQ 461		100% de identidad a la SEQ ID NO 461 del			

		documento WO 2009026558			



Human Antibodies to PCSK9

Web Noticias Imágenes Vídeos Más Herramientas de búsqueda

Cualquier país Cualquier idioma Antes del 15 sept. 2011 Ordenar

Artículos académicos para Human Antibodies to PCSK9
High affinity **human antibodies to PCSK9** ... Sleaman - Mencionado por 18 ... of a monoclonal **antibody** to **PCSK9** on LDL cholesterol - Stein - Mencionado por 311 ... 145, a Monoclonal **Antibody** to Proprotein Convertase ... - Raal - Mencionado por 241

PCSK9 (human) Polyclonal Antibody || Cayman Chemical ...
https://www.caymanchem.com/catalog/10007185 - Traducir esta página
9 dic. 2005 - Antigen: synthetic peptide from **human PCSK9** amino acids 490-502 ... Cayman's **PCSK9** polyclonal **antibody** detects mainly the mature form of the protein ...

PCSK9 Polyclonal Antibody || Cayman Chemical | Supplier
https://www.caymanchem.com/catalog/10240 - Traducir esta página
1 feb. 2007 - **Human PCSK9** precursor is 692 amino acids in length with an estimated molecular weight of 74 kDa. This proprotein is self-cleaved to form a mature protein of ...

A PCSK9-binding antibody that structurally mimics the EGF ...
www.ncbi.nlm.nih.gov/pubmed/20959875 - Traducir esta página
por YG Ni - 2011 - Mencionado por 51 - Artículos relacionados
19 oct. 2010 - We have identified a fragment antigen-binding (Fab) 1D05 which binds **PCSK9** with nanomolar affinity. The fully **human antibody** 1D05-IgG2 completely blocks ...

Patent US20100166768 - High Affinity Human Antibodies to ...
https://www.google.com/patents/US20100166768 - Traducir esta página
1 jul. 2010 - High Affinity **Human Antibodies to PCSK9** US 20100166768 A1 ... Abstract. An human antibody or antigen-binding fragment of a human antibody that specifically ...

Human Proprotein Convertase 9/PCSK9 Antibody
www.rndsystems.com/Products/AF3888 - Traducir esta página
1 feb. 2001 - Specificity Detects **human PCSK9** in direct ELISAs and Western blots. In direct ELISAs, approximately 30% cross-reactivity with recombinant mouse **PCSK9** is ...

Proprotein Convertase 9/PCSK9 - R&D Systems - Products
www.rndsystems.com › Products - Traducir esta página
5 abr. 2007 - **Human** (22) ... Proprotein Convertase 9/**PCSK9**. Products.
DESCRIPTION CATALOG# PRICE
Human Proprotein Convertase 9/PCSK9 ... DPC900 ... $519 ...
Human Proprotein Convertase 9/PCSK9 ... PDPC900 ... Please Inquire ...

Anti- PCSK9 Antibody Products | Biocompare.com
www.biocompare.com/pfu/.../**Antibodies/PCSK9** - Traducir esta página
6 may. 2007 - **Anti-PCSK9 Antibody** Products at Biocompare.com. Compare specifications ... Goat **Anti-Human PCSK9** (C Terminus), (C Terminus) ... 1 2 3 4 5 ...

Full Text - Proceedings of the National Academy of Sciences
www.pnas.org/content/106/24/9820.long - Traducir esta página
por JCY Chan - 2009 - Mencionado por 214 - Artículos relacionados
16 jun. 2009 - Mice engineered to express arrays of fully **human** IgGκ and IgGλ **antibodies** were immunized with soluble, full-length, mature **human PCSK9** (huPCSK9) ...

wo/2010/077854 high affinity human antibodies to pcsk9
https://patentscope.wipo.int/.../detail.jsf?... - Traducir esta página
6 jul. 2010 - An **human antibody** or antigen-binding fragment of a **human antibody** that specifically binds and inhibits **human** proprotein convertase subtilisin/kexin type 9 ...

PCSK9 / NARC1 Antibody, Mouse MAb - Sino Biological Inc.
www.sinobiological.com/**PCSK9-Antibody**-g-510.ht... - Traducir esta página
21 mar. 2010 - Immunogen : Recombinant **Human PCSK9** protein (Catalog#10594-H08H). Clone ID : 4F10H882. Ig Type : Mouse IgG1. Concentration : Formulation : 0.2 um ...

Sugerencia: Buscar solo resultados en **español**. Puedes especificar el idioma de búsqueda en Configuración de Google Académico.

High affinity **human antibodies** to **PCSK9**

MW Sleeman, JH Martin, TT Huang... - US Patent ..., 2013 - Google Patents

An **human antibody** or antigen-binding fragment of a **human antibody** that specifically binds and inhibits **human** proprotein convertase subtilisin/kexin type 9 (hPCSK9) characterized by the ability to reduce serum LDL cholesterol by 40-80% over a 24, 60 or 90 day period ...

Citado por 16 Artículos relacionados Las 5 versiones Citar Guardar

Effect of a monoclonal **antibody** to **PCSK9** on LDL cholesterol

my-pharm.ac.jp [PDF]

EA Stein, S Mellis, GD Yancopoulos... - ... England Journal of ..., 2012 - Mass Medical Soc

... in LDL cholesterol levels after the administration of REGN727 in **humans** supports previous ... LDL-lowering effect, might affect the duration of action of therapeutic **antibodies** because higher ... In summary, we evaluated the effects of REGN727, a fully **human** monoclonal **antibody** ...

Citado por 311 Artículos relacionados Las 16 versiones Citar Guardar

Low-Density Lipoprotein Cholesterol–Lowering Effects of AMG 145, a Monoclonal **Antibody** to Proprotein Convertase Subtilisin/Kexin Type 9 Serine Protease in ...

ahajournals.org [HTML]

F Raal, R Scott, R Somaratne, I Bridges, G Li... - Circulation, 2012 - Am Heart Assoc

... with a monoclonal **antibody** has recently been shown to be effective in lowering LDL-C in **humans**. 12–14 AMG 145 is a fully **human** monoclonal **antibody** to **PCSK9** that yielded LDL-C ... Binding and neutralizing anti–AMG 145 **antibodies** were assayed as reported previously. 15. ...

Citado por 241 Artículos relacionados Las 8 versiones Citar Guardar

Human antibodies derived from immunized xenomice

R Kucherlapati, A Jakobovits, S Klapholz... - US Patent ..., 2000 - Google Patents

... Such **antibodies** tend to be immunogenic when used in **humans**. ... to a recombinant host cell-which is modified to contain the gene encoding either the **human** immunoglobulin with ... In still other aspects, the invention is directed to **antibodies** or **antibody** analogs prepared by the ...

Citado por 283 Artículos relacionados Las 4 versiones Citar Guardar

A proprotein convertase subtilisin/kexin type 9 neutralizing **antibody** reduces serum cholesterol in mice and nonhuman primates

pnas.org [HTML]

JCY Chan, DE Piper, Q Cao, D Liu... - Proceedings of the ..., 2009 - National Acad Sciences

... Mice engineered to express arrays of fully **human** IgGκ and IgGλ **antibodies** were immunized with soluble, full-length, mature **human PCSK9** (huPCSK9). ... a panel of the most potent lines was subcloned for further characterization, yielding the **antibody** termed "mAb1 ...

Citado por 217 Artículos relacionados Las 13 versiones Citar Guardar

A **PCSK9**-binding **antibody** that structurally mimics the EGF (A) domain of LDL-receptor reduces LDL cholesterol in vivo

jlr.org [HTML]

YG Ni, S Di Marco, JH Condra, LB Peterson... - Journal of lipid ..., 2011 - ASBMB

... in which plasma lipid and **PCSK9** profiles are comparable to those of **humans**. 1D05-IgG2 ... elevated risk of CHD (1, 2). Conversely, approximately 2%–3% of the **human** population is ... of **PCSK9** by recombinant LDLr fragments (12–14) or by mono- or polyclonal **antibodies** (15, 16 ...

Citado por 84 Artículos relacionados Las 7 versiones Citar Guardar

Human antibodies derived from immunized xenomice

R Kucherlapati, A Jakobovits, DG Brenner... - US Patent ..., 2000 - Google Patents

... Such **antibodies** tend to be immunogenic when used in **humans**. ... to a recombinant host cell which is modified to contain the gene encoding either the **human** immunoglobulin with the ... In still other aspects, the invention is directed to **antibodies** or **antibody** analogs prepared by the ...

Citado por 228 Artículos relacionados Las 2 versiones Citar Guardar

Atorvastatin with or without an **antibody** to **PCSK9** in primary hypercholesterolemia

researchgate.net [PDF]

EM Roth, JM McKenney, C Hanotin... - New England Journal ..., 2012 - Mass Medical Soc

... (2014) Efficacy and safety of evolocumab (AMG 145), a fully **human** monoclonal **antibody** to ... (2014) Reduction of Low-Density Lipoprotein Cholesterol by Monoclonal **Antibody** Inhibition of **PCSK9**. Annual Review of Medicine 65:1, 417-431, 26. ... (2014) **Antibodies** to watch in 2014. ...

Citado por 235 Artículos relacionados Las 8 versiones Citar Guardar

[HTML] Molecular characterization of loss-of-function mutations in **PCSK9** and identification of a compound heterozygote

sciencedirect.com [HTML]

Z Zhao, Y Tuakli-Wosornu, TA Lagace, L Kinch... - ... Journal of **Human** ..., 2006 - Elsevier

... Whereas gain-of-function mutations in **PCSK9** in **humans** are apparently rare, a spectrum of more-frequent loss-of-function ... Material. Rabbit polyclonal **antibodies** against full-length recombinant **human PCSK9** (6389) and the catalytic domain of **human PCSK9** (295A) were ...

Citado por 276 Artículos relacionados Las 10 versiones Citar Guardar

High-dose atorvastatin causes a rapid sustained increase in **human** serum **PCSK9** and disrupts its correlation with LDL cholesterol

jlr.org [HTML]

G Welder, I Zineh, MA Pacanowski, JS Troutt... - Journal of lipid ..., 2010 - ASBMB

... Therefore, we studied the time course of atorvastatin (80 mg) in **human** subjects ... **PCSK9** mRNA expression (34–36), and our group hypothesized that statin treatment in **humans** should increase ... The exact epitopes recognized by the **antibodies** used in the ELISA are not known at ...

Citado por 114 Artículos relacionados Las 8 versiones Citar Guardar

A Specific Molar Ratio of Stabilizer to Protein is Required for Storage Stability of a Lyophilized Monoclonal Antibody

JEFFREY L. CLELAND,[1] XANTHE LAM,[1] BRENT KENDRICK,[2] JANET YANG,[3] TZUNG-HORNG YANG,[4] DAVID OVERCASHIER,[1] DENNIS BROOKS,[1] CHUNG HSU,[1] JOHN F. CARPENTER[4]

[1]Pharmaceutical R & D, Genentech, Inc., 1 DNA Way, South San Francisco, California 94070

[2]Pharmaceutics Department, Amgen, Inc., Thousand Oaks, California

[3]Quality Control, Genentech, Inc., South San Francisco, California

[4]Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Denver, Colorado

Received 22 June 2000; revised 25 September 2000; accepted 26 September 2000

ABSTRACT: The selection of the appropriate excipient and the amount of excipient required to achieve a 2-year shelf-life is often done by using iso-osmotic concentrations of excipients such as sugars (e.g., 275 mM sucrose or trehalose) and salts. Excipients used for freeze-dried protein formulations are selected for their ability to prevent protein denaturation during the freeze–drying process as well as during storage. Using a model recombinant humanized monoclonal antibody (rhuMAb HER2), we assessed the impact of lyoprotectants, sucrose, and trehalose, alone or in combination with mannitol, on the storage stability at 40°C. Molar ratios of sugar to protein were used, and the stability of the resulting lyophilized formulations was determined by measuring aggregation, deamidation, and oxidation of the reconstituted protein and by infrared (IR) spectroscopy (secondary structure) of the dried protein. A 360:1 molar ratio of lyoprotectant to protein was required for storage stability of the protein, and the sugar concentration was 3–4-fold below the iso-osmotic concentration typically used in formulations. Formulations with combinations of sucrose (20 mM) or trehalose (20 mM) and mannitol (40 mM) had comparable stability to those with sucrose or trehalose alone at 60 mM concentration. A formulation with 60 mM mannitol alone provided slightly less protection during storage than 60 mM sucrose or trehalose. The disaccharide/mannitol formulations also inhibited deamidation during storage to a greater extent than the lyoprotectant formulations alone. The reduction in aggregation and deamidation during storage correlated directly with inhibition of unfolding during lyophilization, as assessed by IR spectroscopy. Thus, it appears that the protein must be retained in its native-like state during freeze–drying to assure storage stability in the dried solid. Long-term studies (23–54 months) performed at 40°C revealed that the appropriate molar ratio of sugar to protein stabilized against aggregation and deamidation for up to 33 months. Therefore, long-term storage at room temperature or above may be achieved by proper selection of the molar ratio and sugar mixture. Overall, a specific sugar/protein molar ratio was sufficient to provide storage stability of rhuMAb HER2.
 J Pharm Sci 90:310–321, 2001
Keywords: protein stability; lyophilization; monoclonal antibody; aggregation; sugars

Correspondence to: J.L. Cleland (Telephone: 650-225-3921; Fax: 650-225-3979; E-mail: cleland@gene.com)

Journal of Pharmaceutical Sciences, Vol. 90, 310–321 (2001)

INTRODUCTION

The successful development of therapeutic proteins depends on the ability to deliver them in

their intact native state. Proteins are often formulated in aqueous solution to allow ease of use, but several degradation processes may be facilitated by the aqueous environment.[1] To obtain the desired stability during storage for up to 2 years, proteins are often dried to reduce the rate of chemical and physical degradation.[2,3]

There have been numerous reports of the ability of sugars, such as sucrose and trehalose, to stabilize proteins during lyophilization and storage in the dried solid.[4–8] Recent studies have also shown the utility of these sugars in producing freeze-dried,[9–11] spray freeze-dried, or spray-dried antibodies.[12,13] The mechanism of stabilization by these sugars during drying has been proposed to occur by producing a glassy matrix to restrict mobility and/or acting as a water substitute (reviewed by Carpenter et al.[14–16]). Several recent cases have shown that a glassy matrix alone is not sufficient to inhibit protein unfolding during dehydration and that hydrogen bonding of the sugar to the dried protein (water substitution) is a requisite for inhibition of lyophilization-induced protein unfolding.[5–7,17,18]

The removal of water in the absence of a stabilizing agent, such as a sugar, often results in a non-native protein structure in the dry state and may lead to aggregation during reconstitution.[4,17,19] Furthermore, it has also been shown with a few proteins that retention of the native protein structure in the initial lyophilized cake is important for optimal long-term storage stability in the dried solid (reviewed by Allison et al.[8] and Carpenter et al.[15,16]). In addition, it has been found that sugars provide a glassy matrix that is required to reduce the rate of degradative reactions during storage in the dried solid.[6,7,9,16,17]

Many stability studies are performed using isoosmotic concentrations of sugars (e.g., 275 mM sugar) and do not determine the minimum concentration required for optimal long-term stability. In the current investigation, to determine the optimum sugar content for protein stability, we studied the effect of a wide range of sugar-to-protein molar ratios, as well as different sugars, on the retention of native protein structure in the dried solid and prevention of degradation during storage. To assess the relationship between protein structure in the dried state and long-term storage stability, protein secondary structure was analyzed by infrared (IR) spectroscopy and the levels of aggregated and deamidated protein were determined in reconstituted samples.

The model protein selected for these studies was the recombinant humanized monoclonal antibody against the human epidermal growth factor receptor-2 (rhuMAb HER2), which has recently been approved by the U.S. Food and Drug Administration for treatment of metastatic breast cancer. This antibody has a molecular weight of ~150 kDa, and is composed of a human Fc domain of the subtype IgG_1 with humanized framework and complementary determining regions (CDRs) designed to provide comparable binding affinity to the murine form (see Carter et al.[20] for details on the humanization process). Previous studies by our group[21] and others[22] have shown that this antibody is susceptible to oxidation at Met-255 and Met-431 in the Fc domain. Deamidation and formation of isoaspartate and succinimide may also occur at Asn30 in the light chain and Asn102 in the heavy chain.[23] These reactions may be slowed or eliminated by the removal of water to produce a freeze-dried product. However, the removal of water may foster denaturation leading to another degradation route, aggregation. A successful formulation of rhuMAb HER2 must therefore provide protection against aggregation during lyophilization and storage, as well inhibition of chemical degradation. In the studies described herein, we have used storage at elevated temperature (40°C) to determine the relative stability of several formulations. We have also investigated conditions that are hypotonic (<275 mM) and may be used for formulations that require dilution into intravenous (iv) saline bags or reconstitution with volumes that are less than the fill volume yielding a more concentrated solution.

MATERIALS AND METHODS

Materials

Recombinant humanized monoclonal antibody against the human epidermal growth factor receptor 2 (rhuMAb HER2) was expressed in Chinese hamster ovary (CHO) cells and purified at Genentech, Inc. The purification was performed using Protein A affinity chromatography followed by cation exchange to remove aggregates and charged forms of the antibody. The final purified antibody was provided in 5 mM sodium histidine, pH 6 by Genentech's Product Recovery Operations.

Methods

Size Exclusion Chromatography

Distribution of intact protein, soluble aggregate and/or fragments of rhuMAb HER2 was determined using size exclusion chromatography (SEC). A high-performance liquid chromatography (HPLC) system (Hewlett Packard 1090L) was used with a TSK 3000 SWXL (Toso Haas Corporation) column at a flow rate of 1 mL/min using pH 7.2 phosphate-buffered saline (PBS) as the mobile phase. A 25-μg sample of protein was injected onto the column and detected by absorbance at 280 nm. Standards of known protein concentration were run as controls for each set of samples. Samples were run twice, and the average value was used to determine the fraction of aggregated protein in the sample [coefficient of variation (CV) $\pm$ 5%]. The fractions of aggregate, intact protein, and protein fragments were determined by assessing the relative peak areas of each species. For SEC, ion-exchange, and hydrophobic interaction methods, three vials were assayed for each time point and the results were reported as a mean and standard deviation (SD). All chromatographic methods yielded complete protein recovery (no protein loss) when compared with appropriate controls.

Cation-Exchange Chromatography

The amounts of deamidated cyclic imide and intact rhuMAb HER2 were quantitatively determined by cation-exchange chromatography (IEX). A wide-pore carboxy sulfon column was heated to 40°C, and chromatography was performed at a flow rate of 1 mL/min. Separation of various charged species was achieved through a NaCl gradient in 20 mM Na phosphate buffer (pH 6.9). Samples were diluted to 1 mg/mL protein, and then 75 μg of protein was injected onto the column and detected by absorbance at 214 nm. All samples were analyzed in duplicate along with appropriate standards (CV $\pm$ 5%).

Hydrophobic Interaction Chromatography

The detection of rhuMAb HER2 oxidized species was achieved by digestion with carboxypeptidase B and papain. The resulting Fab and Fc fragments were analyzed by hydrophobic interaction chromatography (HIC) using a TSK Butyl-NPR column (4.6 $\times$ 35 mm, Tosohaas) and a gradient from 0 to 2 M ammonium sulfate as previously described.[21]

Residual Moisture of Lyophilized Cakes

Residual moisture of lyophilized rhuMAb HER2 was determined by the Karl Fisher (Brinkmann, model 658) titration method. Measurements were made by an electrochemical technique that utilizes the conductive state of a hydranal composite/methanol solution. Samples for residual moisture analysis were prepared by dispersing the lyophilized cake to a powder in the vials. The percentage by weight of residual water moisture present was determined for sample weights ranging from 40 to 70 mg. A mean titer standard (CV $\pm$ 5%) derived from ten 5-μL water samples was used to calculate the percentage by weight of residual water present in the samples. Two or three moisture measurements were conducted per vial depending on the availability of material. Samples for excipient screening all had residual moisture contents of $\leq$ 2% w/w (g water/100 g solid).

pH of Frozen Protein Solution

The pH of rhuMAb HER2 formulated at 25 mg/mL in 60 mM trehalose, 5 mM histidine, pH 6.3, and 0.01% polysorbate 20 was determined at -20°C (frozen) using a combined pH electrode (Mettler Toledo, Wilmington, MA) as described previously.[24] The probe was calibrated at 25, 10, and 0°C with buffer standards at pH 4, 7, and 10 (Baxter Diagnostics), and the SD of the measurements were $\pm$ 0.10 pH units.

Infrared Spectroscopy

The IR spectra of native aqueous protein and protein in lyophilized formulations were acquired and processed as previously described using a Bomem Prota Spectrometer and software.[6–8,18]

Differential Scanning Calorimetry

Differential scanning calorimetric (DSC) analysis of lyophilized formulations was conducted as previously described[6–8,18,25] with a Perkin Elmer DSC-7, and the percentages of crystalline mannitol were determined as detailed previously.[25]

Experimental Conditions

The protein was formulated at 25 mg/mL in either 5 mM succinate, pH 5.0 or 5 mM histidine, pH 6

with 0–250 mM trehalose and 0.01–0.2% w/v polysorbate 20. Additional studies were performed within this range of polysorbate 20 concentrations, and no effect was observed on the stability of rhuMAb HER2 following storage at 40°C for 6 months in formulations containing 60 mM trehalose and 5 mM histidine, pH 6 (data not shown). Additional solutions were prepared at the same protein concentration in 5 mM histidine, pH 6 with 0–250 mM sucrose and 0.1% polysorbate 20. To determine the effects of mannitol on stability of rhuMAb HER2, the protein was also formulated at 25 mg/mL in 5 mM histidine, pH 6 and 0.1% polysorbate 20 with 60 mM mannitol, and with combinations of mannitol and sucrose (55 mM mannitol/5 mM sucrose; 50 mM mannitol/10 mM sucrose; 40 mM mannitol/20 mM sucrose). The effect of protein concentration on stability was determined by formulation of rhuMAb HER2 at 25 or 50 mg/mL in either 60 mM trehalose, 20 mM trehalose/40 mM mannitol, or 20 mM sucrose/40 mM mannitol with 5 mM histidine, pH 6 and 0.1% polysorbate 20. Long-term stability of the lyophilized protein at 40°C was also determined for formulations containing trehalose alone or the mannitol/lyoprotectant combinations at 5 mg/mL rhuMAb HER2 in 5 mM histidine, pH 6 and 0.01% polysorbate 20. Each study was performed on a single lot of each formulation.

Lyophilization

For each formulation, 18 mL of solution was filled into depyrogenated, sterile 50-cc vials and lyophilized in a GT20 freeze-dryer. Primary drying was conducted at shelf temperatures of -10–5°C for 40–68 h (greater time for lower temperatures) and secondary drying was performed for 10–12 h at a shelf temperature of 20°C. For residual moisture studies, vials were removed at different times during secondary drying using a special access port to allow maintenance of vacuum. The chamber pressure was maintained constant at 150 mTorr throughout primary and secondary drying. Vials were capped under partial vacuum. Reconstitution was performed using 20 mL of Sterile Bacteriostatic Water for Injection (SBWFI) containing 1.1% benzyl alcohol. SBWFI was chosen to allow multiple withdrawals of the protein from the vial for repeated administration from a single vial (multidose vial). A formulation consisting of 25 mg/mL rhuMAb HER2, 60 mM trehalose, 5 mM histidine, pH 6, and 0.01% polysorbate 20 was placed at 40°C for 6 months, and vials were reconstituted with either sterile water for injection (SWFI) or SBWFI. This study demonstrated that reconstitution with SBWFI or SWFI yielded equivalent stability.

Stability Assessment

Control samples of each formulation before lyophilization were retained (stored frozen) and analyzed at the same time as lyophilized samples. Each formulation was reconstituted and tested immediately after lyophilization. Each formulation was stored at 40°C for 1 and 3 months in the lyophilized state and then reconstituted and analyzed. Long-term stability (23–55 months) was determined for selected formulations.

RESULTS AND DISCUSSION

A variety of different sugars and molar ratios of sugar to protein were used to produce lyophilized rhuMAb HER2 formulations. As noted in Table 1, after lyophilization and immediate reconstitution (no storage), even in the absence of sugars, there was only a slight increase in aggregate formation. However, on storage of these lyophilized formulations at 40°C, the extent of aggregation increased dramatically (Figures 1 and 2). Without sugars, the extent of rhuMAb HER2 aggregation increased from $\sim$1% to $>$10% after 3 months at 40°C. In the absence of sugar, a greater extent of aggregation was observed in the histidine formulation than in the succinate formulation after 3 months at 40°C (Figure 1). Previous studies had indicated the potential for histidine to provide stabilizing effects during lyophilization and storage,[26] but this stabilization was not observed with rhuMAb HER2.

The addition of sucrose or trehalose in the formulations reduced the amount of aggregation noted after storage and reconstitution (Figures 1 and 2). There was no apparent difference in the stabilizing affect of trehalose or sucrose (Figure 2b). The amount of aggregated protein decreased with increasing sugar concentration up to 100 mM, and only minor reductions in the amount of aggregate over 3 months of storage at 40°C were observed for formulations containing $>$60 mM sugar. At 60 mM sugar, the molar ratio of sugar to protein was 360. Similar stoichiometric amounts of carbohydrates were required to stabilize another recombinant antibody during spray drying and subsequent storage.[12]

Table 1. Effect of Lyophilization on the Aggregation of rhuMAb HER2[a]

Formulation	% Aggregate	
	Before Lyophilization	After Lyophilization
5 mM Succinate, pH 5	0.2 ± 0.1	1.4 ± 0.2
+ 60 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 100 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 150 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 200 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 250 mM trehalose	0.7 ± 0.2	0.9 ± 0.3
5 mM Histidine, pH 6	0.4 ± 0.2	1.1 ± 0.3
+ 30 mM trehalose	0.3 ± 0.1	0.5 ± 0.2
+ 60 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 100 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 150 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 200 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 250 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
5 mM Histidine, pH 6	—	—
+ 60 mM sucrose	0.5 ± 0.1	1.0 ± 0.1
+ 100 mM sucrose	0.4 ± 0.2	0.3 ± 0.1
+ 150 mM sucrose	0.2 ± 0.1	0.6 ± 0.2
+ 200 mM sucrose	0.7 ± 0.1	0.4 ± 0.2
+ 250 mM sucrose	0.6 ± 0.2	0.5 ± 0.2
5 mM Histidine, pH 6		
+ 60 mM mannitol	0.5 ± 0.2	0.4 ± 0.1
+ 55 mM mannitol/5 mM sucrose	0.4 ± 0.3	0.9 ± 0.2
+ 50 mM mannitol/10 mM sucrose	0.0 ± 0.0	0.5 ± 0.1
+ 40 mM mannitol/20 mM sucrose	0.0 ± 0.0	0.0 ± 0.0

[a] All formulations consisted of 25 mg/mL protein and were lyophilized as described in the MATERIALS AND METHODS section.

To determine if this stabilizing phenomenon was specific to sucrose and trehalose, mannitol was used in the formulation buffer at 60 mM (the apparent minimum sugar concentration required for stability). Although storage of the mannitol formulation at 40°C for 3 months resulted in less aggregation than the formulations without sugar, the mannitol formulation provided slightly less protection against aggregation than the same concentration of sucrose or trehalose (Figures 1–3). Previous studies have indicated that mannitol may crystallize during lyophilization, forming a separate phase from the protein.[6,27–19] If this phase separation had occurred, lyophilization-induced unfolding may not be inhibited (vide infra) and the amount of aggregated protein in the mannitol formulations should be comparable to or greater than that in the formulations without sugar. However, this low concentration of mannitol may remain amorphous and in the same phase as the protein during drying, providing protection to the protein. In support of this suggestion, DSC analysis (cf., Carpenter et al.[25]) determined that mannitol in the lyophilized 60 mM mannitol rhuMAb HER2 formulation was only 7% crystalline (data not shown).

Furthermore, the presence of sugars that remain amorphous during lyophilization (e.g., sucrose) in mannitol formulations can further inhibit mannitol crystallization.[30] Several studies have also shown that combinations of mannitol and an amorphous excipient improve protein stability after lyophilization and storage relative to that noted with mannitol alone.[31–33] To test for this effect, sucrose was added to the formulation to yield a range of sucrose and mannitol/protein molar ratios. The use of 20 mM sucrose (sucrose/protein molar ratio of 120:1), which was not optimum for stability when used alone, and 40 mM mannitol was sufficient to provide stabilization against aggregation after storage and reconstitution, yielding comparable stability to 60 mM

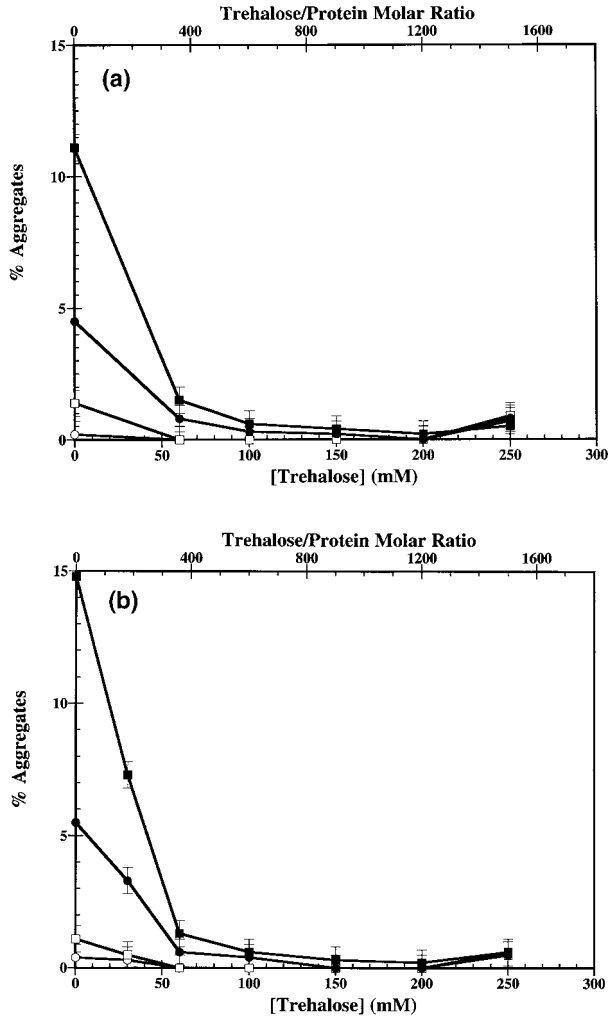


Figure 1. The effect of trehalose concentration on the fraction of aggregated protein after reconstitution of lyophilized formulations was measured by SEC. rhuMAb HER2 was formulated at 25 mg/mL in either 5 mM succinate, pH 5 (A) or 5 mM histidine, pH 6 (B). Samples were analyzed prior to lyophilization (open circles), immediately after lyophilization (open squares), or after storage of the lyophilized protein for 1 (closed circles) or 3 (closed squares) months at 40°C.

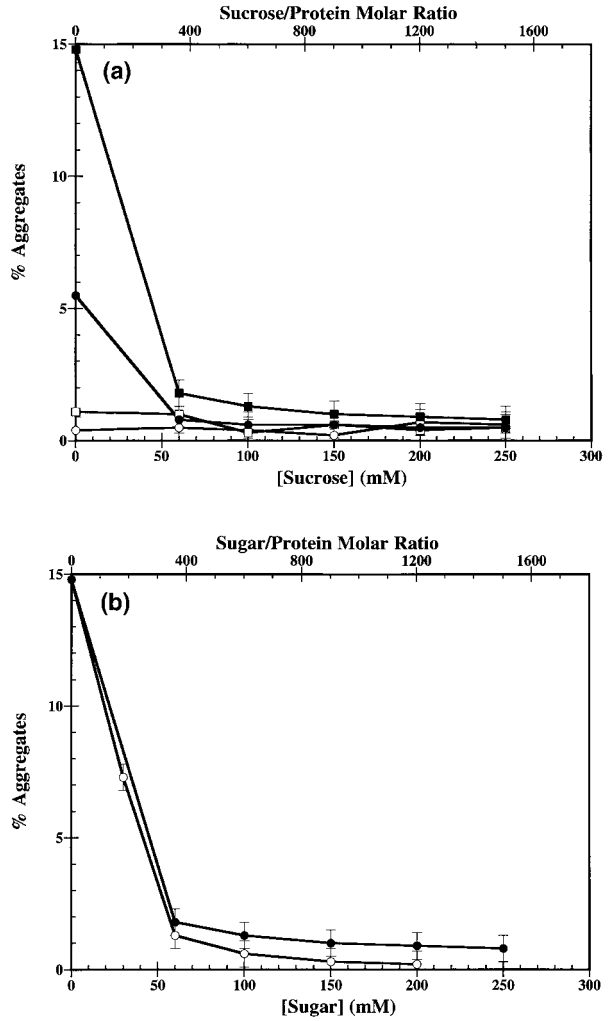


Figure 2. The effect of sucrose concentration on the fraction of aggregated protein after reconstitution of lyophilized formulations was measured by SEC. rhuMAb HER2 was formulated at 25 mg/mL in 5 mM histidine, pH 6. (A) Samples were analyzed prior to lyophilization (open circles), immediately after lyophilization (open squares), or after storage of the lyophilized protein for 1 (closed circles) or 3 (closed squares) months at 40°C. (B) Sucrose (closed circles) and trehalose (open circles, 5 mM histidine, pH 6) formulations provided comparable protection against aggregation upon stored at 40°C for 3 months.

sucrose alone (Figures 2 and 3). Stabilization of lyophilized proteins formulated with mannitol in combination with glycine,[31,34] sucrose, or trehalose[33] have been previously reported and indicate that mannitol provides some protection if it remains in the amorphous form.[32] These studies suggest that mannitol may either facilitate sucrose stabilization of the protein or directly stabilize the protein when maintained in the protein phase during drying (amorphous form). With the lyophilized 20:40 mM sucrose/mannitol rhuMAb HER2 formulation, DSC analysis indicated that only 4% of the mannitol was crystalline.

The effects observed in these experiments may have resulted from the overall concentration of the sugars used in these studies and not the specific molar ratio of sugar to protein. Different rhuMAb HER2 concentrations were therefore

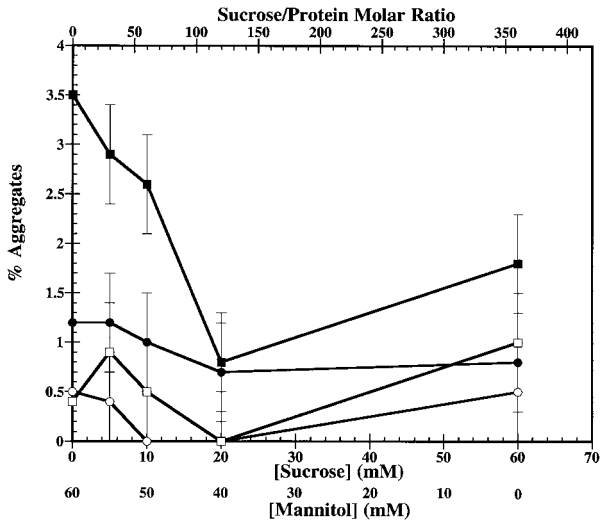


Figure 3. The stability of rhuMAb HER2 formulated in mannitol or mannitol/sucrose formulations was determined by measuring aggregation by SEC after reconstitution of lyophilized formulations. rhuMAb HER2 was formulated at 25 mg/mL in 5 mM histidine, pH 6. Samples were analyzed prior to lyophilization (open circles), immediately after lyophilization (open squares), or after storage of the lyophilized protein for 1 (closed circles) or 3 (closed squares) months at 40°C.

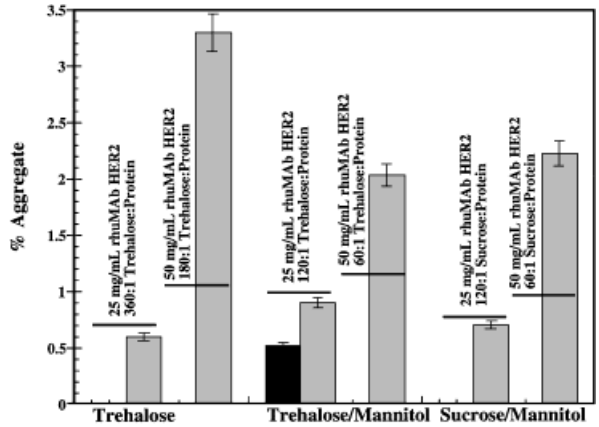


Figure 4. Effect of protein concentration on aggregation of rhuMAb HER2 in lyophilized formulations was assessed after reconstitution either immediately after lyophilization (solid bars) or after storage of the lyophilized protein for 1 month at 40°C.

studied to assess the mechanism of sugar stabilization. Maintaining the trehalose concentration at 60 mM and increasing the protein concentration from 25 to 50 mg/mL resulted in a significant increase in aggregation on storage for only 1 month at 40°C (Figure 4). As a result of this increase in protein concentration, the molar ratio of sugar to protein decreased from 360 to 180. The extent of aggregation at a molar ratio of sugar to protein of 180 was comparable to that observed for 30 mM trehalose and 25 mg/mL rhuMAb HER2 (molar ratio of 180, Figure 1), suggesting that the molar ratio is critical for stabilization of the protein during storage. The mixed sugar systems of 20:40 mM trehalose/mannitol and 20:40 mM sucrose/mannitol yielded similar results where a reduction from a molar ratio of trehalose or sucrose to protein from 120 to 60 yielded a marked increase in aggregation after 1 month of storage at 40°C. Overall, these data revealed that the molar ratio of sugar to protein appears to be a critical variable for protein stabilization during lyophilization and storage.

Long-term protein storage at high temperatures (40°C) was performed on selected samples to further test the impact of the sugar/protein molar ratio on protein stability (Table 2). At very high sugar/protein molar ratios (600–1800:1), only a small increase in aggregation was observed after 33 months at 40°C. At the intermediate molar ratio of 360:1, there was an increase to ~10% aggregation after > 50 months. The rate of aggregation at 40°C for these formulations was ~0.2% per month at 40°C, assuming zero-order kinetics. For a requirement of > 95% monomer at the end of shelf-life, this formulation may be stored at 40°C for 18 months. Decreasing the molar ratio to 180:1 caused a dramatic increase in aggregation (29.9%) after 44 months at 40°C. At the appropriate molar ratio, trehalose and sucrose or their combinations with mannitol may provide long-term, room-temperature stability, and perhaps even formulations that do not require refrigeration even at relatively high ambient temperatures.

Although the initial protein formulations used in the studies just described contained ≤ 2% residual moisture, the impact of 1–8.4% residual moisture was studied for the formulation containing 60 mM trehalose, 5 mM histidine, pH 6, and 0.01% polysorbate 20 to assess the impact of moisture on protein stability. There was no effect of residual moisture over this range on the rate of aggregation during storage at 2–8 or 40°C for 12 months. These data suggest that moisture level differences (1–2% moisture in all formulations) between the formulations did not affect aggregation during storage.

Table 2. Effect of Excipients on the Long-Term Stability of rhuMAb HER2 at 40°C[a]

[Protein] (mg/mL)	Excipient	[Excipient] mM	Molar ratio Excipient: Protein	Storage Time (mo.)	SEC Analysis		IEX Analysis
					% Aggregate	% Monomer	% Main Peak
5	Sucrose	20	600	33	1.1	98.9	80.8
	Mannitol	40	1200				
5	Trehalose	20	600	33	1.7	98.9	81.7
	Mannitol	40	1200				
5	Trehalose	60	1800	33	0.8	99.2	80.1
50	Trehalose	100	300	23	9.7	90.3	76.5
50	Trehalose	60	180	44	29.9	70.1	74.0
25	Trehalose	60	360	51	8.5	91.6	79.8
25	Trehalose[b]	60	360	54	11.0	89.0	76.6
25	Trehalose[c]	60	360	—	0.1	99.9	80.5

[a] All formulations contained 5 mM histidine, pH 6.0, unless otherwise noted. Because of sample limitations, single vials were assayed in duplicate and the average values are reported (CV ± 4%).
[b] Formulation was prepared in 5 mM succinate, pH 5.0.
[c] Reference standard stored at 2–8°C.

Secondary Structure of Lyophilized Protein

To determine the relationship between protein structure in the dried solid and protein stability during storage, the effects of excipients on the secondary structure of lyophilized rhuMAb HER2 (25-mg/mL formulations) were investigated with second-derivative IR spectroscopic analysis. As expected for a monoclonal antibody, the spectra for the aqueous native protein, in the conformationally sensitive amide I region, was dominated by bands at 1640 and 1690 cm^{-1}, which are due to the β-sheet structure (Figure 5). Bands were also observed at 1665 and 1680 cm^{-1}, which are due to the turn structure. When the protein was lyophilized in the absence of sugar, the resulting IR spectra in the dried solid indicated a substantial perturbation of secondary structure, which can be seen as a large decrease in the intensity, a broadening of the band at 1640 cm^{-1}, and a shift in the high frequency β-sheet band to 1695 cm^{-1} and an increase its area (Figure 5). In the presence of 60 mM sucrose, these transitions were partially inhibited, as indicated by the increase in intensity at 1640 cm^{-1}, a shift in the high-frequency β-sheet band to 1693 cm^{-1}, and a decrease in area of this band. In the presence of 60 mM mannitol, the degree of structural protection was slightly less than that noted for 60 mM sucrose (Figure 5). The sample with 20 mM sucrose and 40 mM mannitol had a spectrum that was also very similar to that for the sample with 60 mM sucrose (data not shown). With sucrose alone, the maximal degree of structural protection was obtained with a 100 mM concentration, based on absorbance at 1640 cm^{-1} (Figure 5). Increasing sucrose concentrations to 200 mM did not result in further increases in structural protection (data not shown). Similar results were noted with trehalose, where the maximal degree of retention of native structure also occurred at 60 mM initial sugar concentration (data not shown).

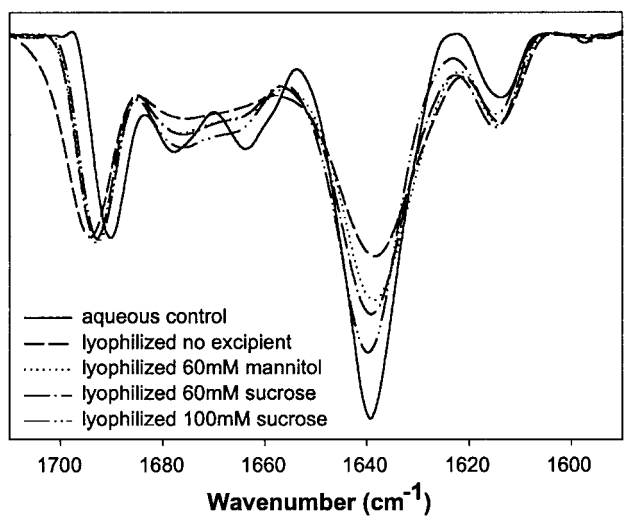


Figure 5. Second-derivative IR spectra were obtained for rhuMAb HER2 formulations: liquid control (5 mM histidine, pH 6, 60 mM trehalose), and lyophilized solids (5 mM histidine, pH 6 without sugar or with 60 mM sucrose, 100 mM sucrose, or 60 mM mannitol).

The spectra of all the formulations taken after 3 months of storage at 40°C, had only slightly reduced absorbance at 1640 cm^{-1} relative to that noted in the respective formulations immediately after lyophilization (data not shown). Also, in the spectra for the stored samples, as was the case for the spectra of samples immediately after lyophilization, bands indicative of intermolecular β-sheet structure (e.g., 1622 cm^{-1}) were not present. Similar results were found with the long-term stored samples that were analyzed by IR spectroscopy (data not shown), which included all formulations shown in Table 2, except the trehalose sample with an excipient/protein molar ratio of 180:1. Unfortunately, the single vial of this sample, which had the highest level of aggregation noted in the current study, was reconstituted before investigation of the dried solid by IR spectroscopy. Taken together, the IR spectroscopic and aggregation results document that either aggregation was not occurring in the dried solid and/or that the level of intermolecular β-sheet present in samples containing up to 15% aggregated rhuMAb HER2 is not sufficient to be detected by solid-state IR spectroscopy.

The capacities of the sugars to inhibit protein unfolding during lyophilization and aggregation during storage/rehydration were almost maximal at 60 mM initial sugar concentration; only a slight improvement is noted by increasing concentration to 100 mM sucrose concentration (Figures 2b and 5). Thus, it appears that optimal storage stability noted with a minimal excipient/protein molar ratio of 360 is due in large part to the retention of native protein structure in the initial lyophilized solid.

Another physical factor that has been found to be important in determining the stability of lyophilized protein formulations during long-term storage is the glass transition temperature (T_g) of the amorphous phase containing the protein.[6–9,14–17] Storage of samples above T_g can lead to rapid degradation.[6–9,14–17] The T_g values for a limited number of the formulations, for which stability results are presented in Figures 1 and 2, were determined by DSC. In all cases, the measured T_g values were >60°C, which far exceeds the storage temperature of 40°C used in the current study.

Chemical Stability of Lyophilized Protein

Another important degradation route for rhuMAb HER2, deamidation and succinimide formation, may be affected by lyophilization in different sugars. Both of these reactions may be catalyzed by water and, therefore, studies were performed with a range of residual moisture levels (1–8.4% w/w) for lyophilized rhuMAb HER2 formulated at 25 mg/mL in 60 mM trehalose, 5 mM histidine, pH 6, and 0.01% polysorbate 20. During storage for 12 months at 40°C, residual moistures between 1 and 4% w/w did not have an impact on the rate or extent of loss in rhuMAb HER2 main peak (only 2% loss in main peak), as measured by IEX. At 8.4% w/w residual moisture, the rate of degradation was increased, yielding a 19% decrease in main peak after 12 months at 40°C. This deamidation may be the result of water catalysis or a reduction in the T_g of the lyophilized cake. Measurements of the frozen pH of the same formulation indicated that the pH changed from 6.3 (solution) to 6.7 on freezing to -20°C. However, the short exposure of the protein to this small pH shift is not sufficient to increase the rate of rhuMAb HER2 deamidation in the lyophilized cake.

When sugars were not present in the formulation, a loss in rhuMAb HER2 main peak in the IEX chromatogram was observed immediately after lyophilization and additional losses were observed on incubation of the lyophilized cake at 40°C (Figure 6). The addition of sucrose (Figure 6a) or trehalose (Figure 6b) reduced the rate of loss in rhuMAb HER2 main peak, and a 60 mM concentration of either was sufficient for maximal protection. Interestingly, when the sucrose/mannitol formulations were tested at these conditions, the 20 mM sucrose/40 mM mannitol formulation prevented a decrease in main peak over 3 months of storage at 40°C compared with ~1% or 5% decrease in main peak for the 60 mM sucrose or mannitol, respectively. The rate of deamidation in the 60 mM sucrose and 20:40 mM sucrose/mannitol formulations are similar, and 60 mM of sucrose or trehalose is sufficient for maximum inhibition of deamidation. Given that these formulations have a similar of retention of native protein structure in the initial lyophilized formulations, the stabilization against deamidation may be caused by maintenance of the protein in the native state.

Longer term studies at 40°C using a range of molar ratios also suggested that a specific molar ratio of sugar to protein inhibits the rate of deamidation. As shown in Table 2, high molar ratios of sugars to protein (600–1800:1) completely inhibited deamidation for 33 months at 40°C. At a 360:1 molar ratio of trehalose to protein, only a

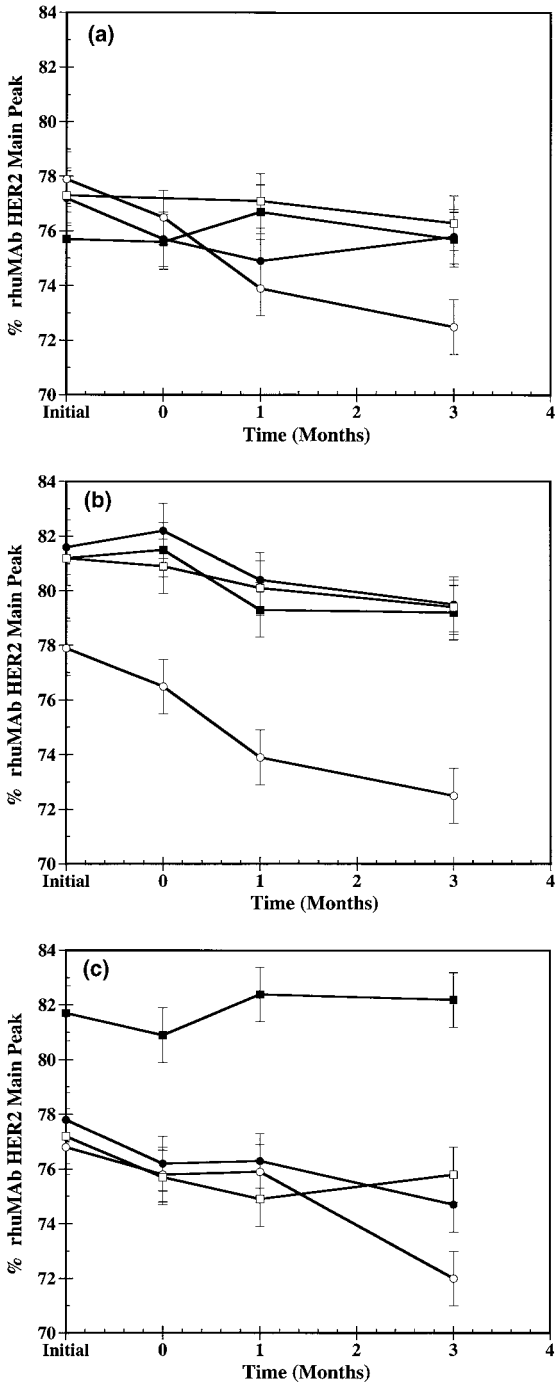


Figure 6. The rhuMAb HER2 main peak area was measured by IEX prior to lyophilization (initial) and after lyophilization with and without storage at 40°C. Formulations consisted of 5 mM histidine (pH 6) and 0.01% polysorbate 20 with 60 (closed circles), 100 (open squares), or 200 (closed squares) mM sucrose (A) or trehalose (B), or without sugar (open circles). (C) Protein in the same buffer with 60 mM mannitol (open circles), 10 mM sucrose/50 mM mannitol (closed circles), 20 mM sucrose/40 mM mannitol (closed squares), and 60 mM sucrose (open squares) was also tested.

slight decrease in main peak was observed after 51 months at 40°C. The lower molar ratio (180:1) resulted in a decrease in main peak of ~6%. The effect of sugars on stabilizing the protein against deamidation was not as significant as their effect on aggregation. Nevertheless, a specific molar ratio of sugar to protein did provide optimum long-term stability.

Each formulation studied in the aggregation and deamidation experiments was also studied for methionine oxidation, which has been previously observed for this protein.[21] After lyophilization and storage at 40°C for 3 months, there was no change in the amount of methionine oxidation (4% oxidized Fc domain), suggesting that this chemical degradation route is effectively inhibited in the lyophilized formulations.

CONCLUSIONS

These studies demonstrated that a minimal specific molar ratio of sugar to protein is sufficient to provide optimal stabilization against aggregation during storage of lyophilized rhuMAb HER2 formulations. A sugar-to-protein molar ratio of 360:1 was sufficient to inhibit aggregation and deamidation after 3 months of storage at 40°C. Both sucrose and trehalose provided equivalent protection during lyophilization and storage. Mannitol alone at the same molar ratio was only slightly less effective at preventing aggregation during storage at 40°C. Mannitol conferred stability most likely because it remained mostly amorphous during lyophilization. The addition of sucrose or trehalose to the mannitol formulations yielded equivalent storage stability compared with both sugars alone at the same total molar concentration (60 mM) and molar ratio (360:1 protein/sugar). Protein stability after storage for several years at 40°C was achieved at molar ratios of 360:1 or greater, indicating the potential for development of room-temperature stable formulations by selection of the appropriate sugar and molar ratio.

The reduction in aggregation and deamidation during storage correlated directly with inhibition of unfolding during lyophilization. Thus, it appears that the protein must be retained in its native-like state during freeze–drying to assure storage stability in the dried solid. This condition may be achieved by selecting the appropriate concentration (molar ratio) of stabilizing sugars for a particular protein.

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