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SUPERINTEND ENCIA DE INDUSTRJA Y COMERCIO

TrÆmite: 011 Evento: 379 Actuación 444

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Act 444 RESPUESTAREQUE Folios: 26

Referenda : Expediente No. 04.057.564
Asunto : Solicitud de Patente para PREPARACION
LIOFILIZADA QUE CONTIENE UN ANTICUERPO
CONTRA EL RECEPTOR EG F
Solicitante : MERCK PATENT GMBH
Fecha de Solicitud : 18 de junlo de 2004

1. Yo, Emilio FERRERO, obrando como apoderado del solicitante en el asunto antes identificado, me refiero al Oficlo No. 13593 notificado por fijación en lista del 26 de

octubre de 2007.

2. Mediante el Oficlo No. 13593 se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de

patente.

Atiendo a las observaciones de la manera siguiente:

2.1. CLARIDAD DE LA SOLIC1TUD

Para subsanar las objeciones que sobre claridad hizo su Despacho, presento nuevo capItulo reivindicatorlo Con 9 reivindicaciones en el cual se han hecho los cambios respectivos, sugeridos por su Despacho. Las modificaciones realizadas fueron los

siguientes:

NIT 860.041.367-3

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- La nueva reivindicación 1 ha sido limitada a los anticuerpos EGFR, los cuales se
encuentran plenamente sustentados en el pliego descriptivo.

- Las reivindicaciones 12 a 15 han sido eliminadas de este pliego reivindicatorio.

- El tØrmino EMD 72000 ha sido reemplazado por Matuzumab.

A este respecto, respetuosamente ponemos de presente que el tØrmino EMD
72000 (h425) es un anticuerpo anti- EGFR humanizado derivado de murino MAB 425 cuya region constante estÆ compuesta por una cadena k y una cadena y-1 humana (ver pÆgina2, primer pØrrafo de la memoria descriptiva).

Adicionalmente el significado, la composición y el mØtodo de obtención del MAB 425, se encuentran sustentados en el cuarto pÆrrafo de la pØgina 1 de la memoria descriptiva.

- Por otra parte, el ingrediente activo se ha limitado a Cetuximab o Matuzumab; los
surfactante se han limitado a polisorbatos y los amino azcicares fueron eliminados de este pliego reivindicatorio. Lo anterior por cuanto (i) los anticuerpos EGFR pueden variar ampliamente en su estructura y propiedades fIsico-quImicas; (ii) el tØrmino surfactante es un tØrmino muy amplio que comprende una gran cantidad
de surfactantes que tienen una estructura y propiedades fisico qulmicas muy diferentes; y (iii) su distinta estructura en comparaciOn a los azCicares, respectivamente.

De esta manera es claro que con las anteriores modificaciones del capItulo reivindicatorio no solamente queda subsanada la objeción que por claridad hizo su Despacho, sino que al haberse limitado el alcance de la protecciOn solicitada, la misma es fÆcilmente determinable por un tØcnico medio y por ende, el mismo estarIa en capacidad de

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compararla frente al estado del arte, de acuerdo con las disposiciones del artIculo 30 de la

Decision 486.

2.2. NIVEL INVENTIVO

A este respecto, es importante poner de presente que el examen de nivel inventivo estÆ
dirigido a determinar si Ia invención reivindicada habla sido sugerida o ensefiada en el estado del arte, de acuerdo con el conocimiento de un tØcnico medio en la materia. Para los anteriores efectos, el mØtodo problema-solución le indica al Examinador que al realizar el examen de nivel inventivo debe verificar las enseæanzas del arte previo sin adoptar la posicion de un inventor que se preguntarla cómo solucionar el problema tØcnico en

cuestión.

Lo anterior, por cuanto si se adopta la posición del inventory se tiene conocimiento tanto del arte previo como de la forma como efectivamente se solucionó el problema tØcnico, la invención en todos los casos le resultarÆobvia y evidente.

Adicionalmente, se debe tener en cuenta que el mericionado mØtodo problema-solución tambiØn seæala que los documentos del estado del arte frente a los cuales se debe efectuar el examen de nivel inventivo son aquellos que dentro del mismo campo tØcnico estØn dirigidos a solucionar el mismo problema. Lo anterior, por cuanto estos son los documentos que se presume debieron ser de conocimiento del inventor para a partir de ellos formular su invención. AsI los documentos relacionados con otro campo tØcnico o dirigidos a solucionar cualquier otro problema no serlan relevantes para estos efectos en la medida que no harlan parte del grupo de documentos de los cuales el inventor hubiera podido derivar de manera obvia y evidente su invención.

De acuerdo con lo anterior, procederemos a indicar las razones por las cuales los documentos citados no afectan el nivel inventivo de Ia presente solicitud de patente. Veamos:

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Ese Despacho afirma que el documento Dl afecta el nivel inventivo de la presente invención pues considera que las formulaciones objeto de la presente invención se derivan evidentemente de la divulgación efectuada en dicho documento y que hubiesen podido ser desarrolladas por cualquier tØcnico con conocimientos bÆsicos enFarmacotØcnia.

Este documento estÆ dirigido a formulaciones que contienen anticuerpos, pero solo se revela en detalle las formulaciones para algunos anticuerpos. AsI, se encuentran revelados especIficamente las formulaciones que comprenden anticuerpos monoclonales dirigidos contra el virus de la Hepatitis B (AK HBV) (Ejemplos 1, 2, 2a, 3, 4, 5, 6, 7, 8, 9). Una preparación que comprende el anticuerpo en contra de la L-selectina (Anti-L-selectina) (Ejemplo 10). Y otra preparación que comprende un anticuerpo contra el receptor del factor de crecimiento del nervio L (anti-LNGFR) (Ejemplo 11).

Sin embargo, en vista de que la estabilidad de dichas formulaciones depende Ønormemente de las propiedades fisico-qulmicas de los ingredientes activos incluidos, y tales propiedades varlan enormemente entre los diferentes anticuerpos dependiendo de su secuencia de protelna, Ia materia descrita o revelada en Dl NO constituye una ensePianza general susceptible de afectar el nivel inventivo cualesquiera otras formulaciones que contienen anticuerpos, tal y como es el caso de la presente solicitud de patente.

AsI entonces, teniendo en cuenta que la formulación de la presente invención difiere de la formulación de Dl en lo que respecta al ingrediente activo y como en el Ejemplo 11 de Dl se revela una formulación que contiene un anticuerpo que estÆ dirigido al factor de crecimiento (nervio L), y Ia formulaciOn de Ia presente invención contiene un anticuerpo que estÆ dirigido a otro factor receptor de crecimiento, es decir, al receptor del factor de crecimiento epidØrmico, de acuerdo con el mØtodo problema-solución el documento Dl puede considerarse como el arte previo mÆscercano de la presente invención.

Sin embargo, se debe tener en cuenta que el problema que fue resuelto por la presente

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invención fue el de proporcionar una preparación estable de anticuerpos dirigidos a EGFR
que no comprendiera auxiliares toxicológicamente aceptables, estable para el almacenamiento bajo condiciones de stress, tales como elevadas temperaturas y humedad
y que no fueran reconstituidas con un solvente acuoso en una solución lista para su uso
con un alto contenido de ingrediente activo; el cual no fue planteado ni resuelto en la anterioridad citada.

De esta forma, el problema ha sido resuelto por la presente solicitud mediante una nueva preparación liofihizada, que comprende, ademÆs de un anticuerpo anti-EGFR, un azicar, un aminoÆcidoy un surfactante. Como se ha demostrado mediante datos comparativos a lo largo de la solicitud, la preparación liofilizada que contiene tales ingredientes de la presente invención tiene una alta estabilidad inesperada en comparación con formulaciones que no contienen todos dichos auxiliares.

Dicho carÆcter inesperado estÆ demostrado porque una persona versada en la materia nunca considerarla las enseiianzas de Dl ni siquiera transferirla dichas enseæanzasa los anticuerpos anti-EGFR, ni tampoco tendria una expectativa razonable para obtener la formulación con tan alta estabilidad silo hubiera considerado.

A este respecto, deseamos resaltar que Dl solamente proporciona datos de estabilidad para el anticuerpo anti-hepatitis, el cual es totalmente diferente de los anticuerpos de la presente invención. Para el segundo anticuerpo (anti-LNGFR, Ejemplo 11) se proporcionan muy pocos e insuficientes datos, ya que no se da información respecto a los agregados, lo cual es uno de los criterios principales para Ia estabilidad de una formulación. Dicho anticuerpo-LNGFR es al arte previo mÆs cercano y en dicho documento no se proporcionaron datos respecto a Ia estabilidad.

Adicionalmente a Ia falta de información de datos respecto a la estabilidad, la información dada con respecto anti-LNGFR, Dl no enselia la transferencia de la formulación revelada

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para este anticuerpo a otros anticuerpos, en el caso especIfico al anticuerpo anti-EGFR,
para el cual si se proporciona en las formulaciones de la presente invención.

AsI mismo, la preparación con anticuerpo anti-LNGFR solo contiene 0.25 mg/ml de anticuerpo pero tiene por otro lado una composiciOn cuantitativa y cualitativa idØntica de
los auxiliares en comparación con los otros ejemplos. Adicionalmente a esto y en contraste a todas las demØs preparaciones reveladas en Dl, no se suministra datos de estabilidad para la preparaciOn que contiene anti-LNGFR. Este es el caso, a pesar de que la preparación contiene tales bajos contenidos de anticuerpo y tales attas cantidades de auxiliares estabilizantes, los cuales, si la preparaciOn funciona, deberIa tener un efecto beneficioso respecto a la estabilidad en el almacenamiento.

Respetuosamente Ilamamos la atenciOn del seæorExaminador al hecho de que si bien el anti-LNGFR al igual que el anti-EGFR estÆn dirigidos al factor de crecimiento, esto no implica caracterIsticas similares en la preservación y preparación de dicha preparación, ya que Ia preparación farmacØutica de la presente invenciOn no contiene ningÆn receptor de crecimiento sino anticuerpos relacionados a ellos. En consecuencia, una persona versada en la materia, que tenga que desarrollar una preparación farmacØutica que contenga un anticuerpo, no considerarla las enseæanzas relacionadas al objetivo, en el cual un anticuerpo estarla unido, pero si aquel que se refiera al anticuerpo coma tal, el cual estarIa presente en la preparación farmacØutica a ser desarrollada.

Par otra parte, el desarrollo de la formulación farmacØutica de un anticuerpo, de las caracterIsticas fIsico-qulmicas del anticuerpo y de sus vIas de degradaciOn son factores decisivos, mÆs no su objetivo farmacolOgico. A pesar del hecho de que ambos anticuerpos, el anti-LNGFR y el anti-EGFR, estÆn dirigidos a diferentes receptores del factor de crecimiento, esto no resulta en una similitud de tales propiedades entre los anti-LNGFR descritos en el Ejemplo 11 de Dl y el anticuerpo anti-EGRF de la presente invención tal coma se describe mÆs abajo. Por lo cual, una persona versada en la materia no considerarIa la informaciOn divulgada en el Ejemplo 11 de Dl, cuando tenga que

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desarrollar una preparación farmacØutica estable en el almacenamiento y que contenga
un anticuerpo anti-EGRF.

Adicionalmente, la estructura del anticuerpo estÆ principalmente determinada por su tipo,
por ejemplo, inmunoglobulina GI, G2, G3, etc., y adicionalmente, por las especies de Ia
cual deriva el anticuerpo, por ejemplo, ratón, rata, humano, quimØrico o humanizado. Aparte de esto, existe la region variable de la cadena pesada y liviana en donde los lados de enlace para el antIgeno son distribuidos. Sin embargo, en relación al anticuerpo en su totalidad, dichos lados de enlace constituyen sOlo una pequeia parte del total de su masa.

Por consiguiente, como es bien conocido, las propiedades fIsico-qulmicas de un anticuerpo dependen principalmente de la estructura primaria del anticuerpo, es decir, de su tipo y especie. AsI, los anti-LNGFR descritos en el documento citado, no proveen informaciOn con respecto al tipo ni especie del anticuerpo del cual se derivan.

Por lo tanto, no existe comparación posible con respecto a otro anticuerpo, incluyendo los anticuerpos objeto de Ia presente invención, por lo cual no se pueden deducir anticuerpos con propiedades fIsico qulmicas similares. Adicionalmente, Dl no divulga informaciOn con respecto a los lados de enlace del anti-LNGFR, es decir, respecto al lado exacto del receptor LNGFR al cual se une el anticuerpo. En vista de que un receptor posee varios epItopes, en contra de los cuales un anticuerpo podrIa dirigirse, no se pueden deducir propiedades fIsico-qulmicas a partir de dicho objetivo.

A partir de lo antes descrito, podemos concluir que el anti-LNGFR descrito en el Ejemplo 11 de Dl fue revelado de una manera tan general que ninguna similitud estructural con respecto a otros anticuerpos podrIa ser deducidas a partir del mismo. Adicionalmente, el simple hecho que el anti-LNGFR y los anticuerpos de la presente invención estÆn dirigidos a receptores del factor de crecimiento diferentes, esto no tiene impacto alguno sobre sus propiedades fi’sico qulmicas y, como resultado de ello, tampoco tendrÆ impacto sobre las preparaciones farmacØuticas desarrolladas que contengan tales anticuerpos.

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Adicionalmente, toda vez que tambiØn es requerido que la preparación farmacØutica a ser desarrollada posea estabilidad en el almacenamiento, existe un punto adicional que mencionar: la degradación de proteInas como los anticuerpos ocurre por varias rutas de degradación, como la de Ia deamidación, oxidación, eliminación-Beta (ver Anexo 3.2. Lai
MC et.al. in Journal of Pharmaceutical Sciences, Vol. 88, No. 5, May 1999.) En vista de que la ruta de degradación relevante para la degradación de la proteIna especIfica (en el presente caso, el anticuerpo) es dependiente de la estructura principal del anti-LNGFR, y considerando que en Dl no se revela absolutamente nada sobre la estructura primaria del anti-LNGFR, no se puede encontrar evidencia alguna en dicho documento con respecto al desarrollo de una preparación farmacØutica estable al almacenamiento que contenga un anticuerpo diferente.

En consecuencia, una persona versada en la materia a partir de las enseIianzas de Dl concluirla que Ia composición de auxiliares con anti-LNGFR proporciona poca o ninguna estabilidad. Por 10 cual, aÆn si una persona versada en la materia considerara el anticuerpo anti-LNGFR por ser similar al anticuerpo anti-EGFR, dicha persona no transferirIa la composición del Ejemplo 11 del documento Dl a cualquier otro anticuerpo receptor del factor de crecimiento a los fines de obtener una preparación farmacØutica de almacenamiento estable y que contenga un anticuerpo receptor del factor de crecimiento

tan distinto.

Finalmente y aunque el seior Examinador no realizó anÆlisis algunosobre las razones por las cuales considera que D2 afecta el nivel inventivo de la presente invención, presumiblemente porque no siendo el estado del arte ma’s cercano no resulta finalmente relevante para estos efectos, respetuosamente nos permitimos poner de presente lo siguiente:

Dicho documento describe el marcaje radial y eficiencia del marcaje radial de un anticuerpo murino anti-EGFR mediante el uso de una formulación congelada al seco que

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contiene tal anticuerpo. La formulación congelada al seco usada para el marcaje radial
fue preparada mediante Ia congelación de una solución que contiene el anticuerpo y varios carbohidratos (trehalosa, glucosa, sorbitol, sacarosa, manitol, inositol) como aditivos y la posterior liofilización por 24 horas. La estabilidad de la formulación fue probada despuØs
del almacenamiento en varias condiciones (a 4°C por 1 aæo; a 37°C por una semana y a 45°C por 24 horas) mediante la determinación de la eficiencia del marcaje de las

formulaciones.

En contraste a la materia de Ia presente invención, la mejora descrita en cuanto a la estabilidad lograda a travØs de la adición de un carbohidrato, se relaciona con la eficiencia del marcaje radial y no con una mejora en la estabilidad de las preparaciones farmacØuticas despuØs de su almacenamiento, como son, la no formación de productos de degradación o agregados despuØs del almacenamiento y reconstitución mediante la adición de un solvente acuoso a los fines de obtener una solución lista para su uso.

Por to tanto, no resulta posible derivar ninguna ensePianza tØcnica de las eriseuianzas de D2 con respecto al problema tØcnico que fue descrito en la presente invención; como lo es, la estabilidad del producto farmacØutico, hecho este que refuerza nuestra conclusion de que se trata de un documento que no hace pane del estado del arte mÆs próximo y que por ende, resulta irrelevante para determinar la patentabilidad de la presente

invención.

En adiciOn, el tiempo de almacenamiento descrito en el documento D2 es definitivamente insuficiente para investigar la estabilidad de una formulación farmacØutica, ya que una la misma, como es de conocimiento de un tØcnico medio en la materia, tiene que cumplir con altos estÆndares deestabilidad farmacØutica.

MÆS atn, en comparación con la presente invención, las enseæanzas del documento D2
con respecto a Ia estabilidad a travØs de la adición de auxiliares, estÆ limitada a

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carbohidratos solamente y no a ninguno de los auxiliares, tales como un aminoÆcido yun
surfactante, tal y como se menciona en la presente invención.

De tal manera que Si se tiene en cuenta que Ia presenda de todos estos auxiliares es,
esencial para poder obtener la inesperada presente formulación altamente estable, el
documento D2 no puede tenerse como una anterioridad vÆlida de la misma capaz de afectar su nivel inventivo. Adicionalmente, no existe enseæanza y/o indicio en D2 que indique que la estabilidad de la formulación puede mejorar con la adición de auxiliares adicionales, razón por la cual la presente invención no se podrIa derivar de aquØl de manera obvia y/o evidente.

En vista de lo antes expuesto, el documento D2 ni anticipa la materia de Ia presente invención, ni da sugerencia con respecto a tal materia. Por lo cual no se puede considerar que sea un documento que afecte de algÆnmodo el riivel inventivo de esta invención.

3. ANEXOS:

3.1. Nuevo pliego reivindicatorio con 9 reivindicaciones;
3.2. Lai MC et.aI. in Journal of Pharmaceutical Sciences, Vol. 88, No. 5, May
1999;
3.3. Formulario de modificaciones;
3.4. Recibo por valor de $103.000 por concepto de modificaciones en solicitud
pendiente.

Seæora Jefe,

EMILIO FERRERO
T.P.A. No. 31.929
COLO-09266-841-010-0

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No. M-2008-117285\NNJ

CAVELIER ABOGADOS COLO 09266-841-010-0 ID-117287

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S U P E R I N T E N D E N C I A

FORMULARO UNICO DE CORRECCIONES Y MODIFICACIONES

0 SOLICITUD DE:

U Corrección de Error Material X Modificaciôn a una solicitud
Nombre: MERCK PATENT GMBH
Sociedad constituida existente bajo las]eyes de

2 Alemania. IDENTIFICACION SOLICITANTE Dirección: Frankfurter Strasse 250,
64293 Darmstadt, C.C. U NIT LI
Alemania C.E. LI Otro [1
Domicilio: Darmstadt,
Alemania NCimero:

TelØfono: 546 09 70 Fax: 249 40 70
E-mail: clientesO-cavelier.com

IDENTIFICACION 0 Nombre: Emillo FERRERO

Direcciôn: Carrera 41 No. 72-35 C.C. tI NIT U

REPRESENTANTE TelØfono: 3473611 Fax: 217 92 11 C E U Otro U o APODERADO E-mail: cavelier@cavelier.com

I Nümero:438.019TP31.929

0 Nümero de expediente: 04.057.564

0 Descripción de la corrección o modificación solicitada:

Presento nuevo capitulo reivindicatorio compuesto por 9 reivindicaciones con elfin de superar las objeciones hechas por Su Despacho a la presente solicitud de Patente solicito que el examen de patentabilidad sea efectuado sobre este nuevo capitulo reivindicatorio.

Comprobante de pago No. 08-5,051 Fecha: 21 de enero de 2008

Anexos

’ " II Comprobante de pago de la tasa por $1 03.000.00 por concepto de modificaciones.
U Poderes, si fuere el caso
U Documento que acredita la existencia representaciOn legal de las partes, cuando sean personas jurIdicas.

8 NOMBRE: EMILIO FERRERO FIRMA:

C.C. 438.019 de Usaquen T.P.A. 31.929

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REIVINDICACIONES

1. Una preparaciOn farmacØutica liofilizada de anticuerpos mono o policlonales que comprende un azUcar 0 Ufl amino azUcar, un aminoÆcido y un surfactante, caracterizada en que el anticuerpo esCetuximab oMatuzumab.

2. La preparaciOn farmacØutica liofihizada de acuerdo a la reivindicaciOn 1, que consiste esencialmente de un anticuerpo, un azücar o amino azUcar, un aminoÆcido,un buffer un surfactante.

3. La preparaciOn farmacØutica liofihizada de acuerdo a la reivindicaciOn I y/o 2, caracterizada en que el azUcar es un mono-, di o trisacÆrido, preferiblemente sacarosa, maltosa o trehalosa.

4. La preparaciOn farmacØutica liofihizada de acuerdo a una o varias de las reivindicaciones I a 3, caracterizada en que al amino azUcar es glucosalina, N-metilglucosamina, galactosamina o Æcidoneuraminico.

5. La preparaciOn farmacØutica liofihizada de acuerdo a una o varias de las reivindicaciones I a 4, caracterizada en que el aminoÆcido es un aminoÆcido bÆsico, un aminoÆcidoÆcidoo neutral, preferiblemente arginina, lisina u ornitina.

6. La preparaciOn farmacØutica liofihizada de acuerdo a una o varias de las reivindicaciones I a 5, caracterizada en que el surfactante es un polisorbato o un p01 Imero polioxietileno-polioxipropileno.

7. La preparaciOn farmacØutica liofihizada de acuerdo a la reivindicaciOn 6, caracterizada en que el surfactante es el ester pohioxietileno sorbitan Æcido graso, polioxietileno (20) sorbitanmonooleato o pohioxietileno (20) sorbitanmonolaurato.

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MINIREVIEW

Solid-State Chemical Stability of Proteins and Peptides

M. C. LAO AND E. M. Topp

Contribution from Department ofPharmaceutical Chemistry, The University of Kansas, 2095 Constant Ave., Lawrence, Kansas 66047.

Received September 16, 1998. Final revised manuscript received March 5, 1999.
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Abstract 0 Peptlde and protein drugs are often formulated in the
solid-state to provide stabilization during storage. However, reactions
can occur in the solid-state, leading to degradation and inactivation
of these agents. This review summarizes the major chemical reactions
affecting proteins and pepildes in the solid-state: deamidaion, peptide bond cleavage, oxidation, the Maillard reaction, fl-elimination, and dimerizatlon/aggregatlon. Physical and chemical factors influencing these reactions are also discussed. These include temperature, moisture content, excipients, and the physical state of the formulation (amorphous vs crystalline). The review Is Intended to serve as an aid for those involved in formulation, and to stimulate further research on the determinants of peptide and protein reactivity In the solid-state.

Introduction

In the last two decades, proteins and peptides have become an Important class of potent therapeutic drugs. However, their susceptibility to chemical degradation In solution presents a challenge in the development ofstable protein pharmaceuticals.’ As a result, many polypeptide drugs are formulated as iyophllized or freeze-dried products to prolong their shelf llfe.24 While a dry" formulation Is generally more stable than the corresponding aqueous formulation, chemical degradation reactions can still occur.?-4 In some cases, protein stability In the solid state Is less than or comparable to that in solution.5’6 Factors that may impact the chemical stability of pro-teins and peptides in the solid-state include residual

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Volume 88, Number 5

moisture and the exciplent(s) used In a formulation. Exciplents, such as polymers, are often Included in protein and peptide formulations to protect the drug from degrada-tion during processing and/or storage or to act as a matrix for controlled release. This review presents an overview of the chemical degradation reactions common to proteins and peptides In the solid state, and of our current knowledge regarding the effects offormulation and storage factors on peptide and protein stability In these systems.
The degradation pathways of proteins In the solid state can be classified Into two types: chemical and physical. Chemical Instability Involves covalent modification of a protein or amino acid residue to produce a new molecule via bond cleavage, bond formation, rearrangement, or substitution. These chemical processes include such reac-tions as deainldatlan at asparagine (Asn) and glutamine (Glu) residues.7 oxidation ofsulfur atoms at cystelne (Cys) and methionine (Met) residues, disulfide exchange at Cys, and hydrolysis of aspartate (Asp) and glutamate (Glu) residues.8 Physical instability refers to changes In the three-dimensional conformational integrity of the protein and does not necessarily involve covalent modification. These physical processes include denaturation, aggrega-tion, precipitation, and adsorption to surfaces.’ Chemical instabilities, such as deamidatlon and disulfide bond cleavage, may lead to physical Instabilities, and vice versa. The physical instabilities of proteins will not be discussed In detail here, since the focus Is chemical Instability. The reader may refer to a recent article on the physical
Instability of proteins for further discussion.9 Instead, this
review will discuss reactions and factors that contribute
to the chemical Instability of proteins and peptides In the
solid-state.

The different types of chemical reactions that contribute

1999, American Chemical SocIety and 10.10214s980374e CCC: $18.00 Journal of Pharmaceutical Sciences I 489 American Pharmaceutical Association Published on Web 04/14/1999 Vol. 88, No. 5, May 1999

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 NH CH--JOH ’> CH2 Cl NHCH’C- NH
0 0 0

lanAzp. 1fappt1d AflnxppIIM
Scheme 1Deamidaflon.

to protein and peptide Instability In the solid-state are first presented. These Include deamidatlon, peptide bond cleav-age, oxidation, the Mallard reaction, -el1m1natlon, and covalent dlxnerlzatlon or aggregation. In the next section, formulation factors that affect chemical stability, such as temperature, moisture, and exclplents, are discussed. The review concludes with a summary and a discussion of the Implications for future research.

Solid-State Reactions of Peptides and Proteins

DeamidationChemical Instability in the solid state due to deamidatlon has been observed for human growth hormone (hG4.410 recombinant human lnterleukln-1 re-ceptor ants Is’ recombinant bovine somatotropin (growth hormone),12 and lnsulin.114 While there have been numerous mechanistic studies of protein and peptide deaxnldatlon In solution.1159 few such studies have been reported for deanildatlon In the solid state. Two studies which provide a mechanistic perspective on deanildatlon In solids are summarized below.

The stability and mechanism of degradation of the Asn-hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala) were studied in solid formulations lyophilized from acidic solutions ranging from pH 3-5. The main degradation pathway for Asn-hexapeptide in the pH 3 formulation is deamidatlon via hydrolysis of the Mn side chain to produce the Asp-hexapeptide, which Is further hydrolyzed at the Asp-Gly amide bond to generate a small quantity of tetrapeptide (Scheme 1).18 As the pH of the solution prior tolyophiliza-tion increases from 3 to 5. Intramolecular attack of the carbonyl center ofthe Mn side chain by the amide nitrogen of the succeeding amino acid to form a cyclic hide Intermediate becomes more prominent, as evidenced by an increase In the cyclic bride in the product distribution (Scheme 1).18 In solution at pH 5, the cyclic hide Is hydrolyzed to form the lsoAsp-hexapeptlde, which is the dominant degradation product;’5 however, the lsoAsp-hexapeptide was not observed In the solid state.18 The absence of the isoAsp-hexapeptlde in the product distribu-tic,-, may be due to the low level of water available for hydrolysis In the solid state, The mechanism of deaxnlda-tion for the Asn-hexapeptide in the solid state was found to be similar to that in solution.18 In an extension of this work, we have recently investigated the deamidatlon ofthis

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peptide In solid poly(vinyl alcohol) and poly(vinyl pyrroli-done) matrixes.20 As In the lyophilized peptide, the mechanism of deamidatlon appears to be similar to that In solution, but the kinetics and product distribution are altered, particularly in matrices of low water content. Human insulin has also been observed to undergo deaxnldatlon In the solid-state via a mechanism similar to that In solution. 13 In Insulin formulations lyophilized from acidic solutions (pH 3-5), the rate-determining first step involves Intramolecular nucleophilic attack of the C-terminal Mn21 carboxylic acid onto the side-chain arnide carbonyl to release ammonium and to form a reactive cyclic anhydride Intermediate, which can further react with various nucleophiles.13 The cyclic anhydride Intermediate may react with water to form [desainidoAzil Insulin,13 and may also react with another molecule of insulin to form covalent dlxners.13 While the cyclic hide Intermediates formed during Asn-hexapeptlde deamidatlon In the solid state were observed to accumulate,n the cyclic anhydride Intermediate formed during Insulin deaxnldatlon did not.13 Strickley and Anderson were able to verify that insulin deamidatlon proceeds via formation of a cyclic anhydride by using aniline trapping of the intermediate.13 Consistent with these findings. Pikal and Rlgsbee observed that the deamidatlon of human Insulin occurs predominantly at ASflA2,. except at high relative humidity when dearnidatlon at Asna3 Is more prevalent. 14
Similar to the degradation of the Asn-hexapeptide discussed previously, the solid-state degradation of a model Asp-hexapeptlde (Val-Tyr-Pro-Asp-Gly-Ala) Is dependent on the pH of the bulk solution prior to lyophillzatlon. This value is often referred to as the pH of the solid, since the true hydrogen Ion activity Is difficult to measure and pH Is technically undefined In the solid-state. Under acidic conditions (’pH" 3.5 and 5.0), the Asp-hexapeptide mainly decomposes to produce a cyclic hide Intermediate via base-catalyzed Intramolecular cydlization.n Hydrolysis of the Asp-Gly amide bond also occurs but to a lesser extent. Under neutral and basic conditions ("p1-I" 6.5 and 8.0), the Asp-hexapeptide degrades exclusively via Intramolecular cyclizatlon to produce the Asu-hexapeptide, which is fur-ther hydrolyzed to form the lsoAsphexapeptIde.n At "pH" 8, the lsoAsp-hexapeptlde Is the dominant degradation product.n similar to that observed In solution.24

Peptide Bond CleavageA second common degrada-tion pathway for peptides and proteins Involves cleavage of the peptide bond. Representative pathways of peptide bond cleavage are shown In Scheme 2. Lyophilized human relaxln formulated with glucose can undergo hydrolytic cleavage of the C-terminal serine (Ser) residue on the B-chain (Trpts-Serts-COOH) upon storage at 40 ’C.25 This observation was supported by a reduction In molecular mass corresponding to the loss of Ser from fragment T5-T9 of relaxln, as verified by liquid chromatography/mass spectroscopy (LCIMS) and tryptic digest.25 Li et al. proposed that this cleavage involved an Initial reaction of the Ser hydroxyl group with glucose followed by subsequent hy-drolysis of the Trp-Ser bond via a cyclic intermediate. In the solid state, the major degradation pathway of aspartame (a-aspartylphenylalanlne methyl ester, APM) Is Intermolecular cydlizatlon to form exclusively diketopip-erazine (DKP) with the elimination of methanol.26 In solution, the degradation of aspartame at neutral and basic pH also occurs mainly via cyclizatlon to form diketopip-erazine (DKP) or hydrolysis at the ester linkage to form a-aspartylphenylalanlne (AP) and methanol.27 Since water was absent In the aspartame solid-state study, no hydroly-products were observed.26
The Instability of the undecapeptide substance P (SF) In the solid state also proceeds through dlketoplperzlne

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Scheme Proteolysts.

formation, The main pathway of decomposition consists of the sequential release of N-terminal dipeptides via their dlketoplperazlnes, cydo(Arg-Pro) and cyclo(Lys-Pro). Un-der the conditions studied, the release of N-terminal dipeptides dominates over other possible routes of spon-taneous modification, such as oxidation and deamldatlon.3’ OxidationThe side chains of His, Met. Cys, Trp, and Tyr residues In proteins are potential sites for oxidation (Scheme 3).’ A major chemical decomposition pathway for human growth hormone (hGH) In the solid state Is me-thionine oxidation at Met4 to form the su]foxlde.’° Even with minimal oxygen (-0.05561) in the vial headspace, decomposition via oxidation is comparable to or greater than that due to the alternative reaction of deamldation.4 Storage of lyophilized hGH In an oxygen atmosphere results In greater decomposition than storage in a nitrogen atmosphere.3’ AsIn solution, atmospheric oxygen can easily oxidize Met residues in the solid state, leading to chemical Instability and loss of biological activity.
Human insulin-like growth factor I (hIGF-I), lyophilized from phosphate buffer, also undergoes oxidation at its Met residue.3’ There were no significant differences In reaction rates (second-order kinetics) between solution and solid states.30 However, Met oxidation in the solid-state consti-tutes a greater fraction of the total protein modification than In solution.30 Both oxygen content and light exposure affect the oxidation rate.30 Exposure to light increases the oxidation rate by a factor of 3030 This Increase In oxidation rate with exposure to light and oxygen suggests that photooxidatlon and molecular oxygen may be involved In the generation ofradicals. However, no further experiments were conducted to determine the nature of the radicals Involved or the mechanism of Met oxidation In the solid state.

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The oxidative dearnidation of a cyclic hexapeptide, ace-stelne-asparagine-(5,5-diniethyl4-thlazolldinecarbo.. nyl)-(4-(anilnomethy])phenylalanlne)-glyclne-aspartic acid-cystelne cyclic disulfide, in a lyophilized mannitol formula-tion does not appear to depend on molecular oxygen as a reactive species,3’ The oxidation reaction occurs at the aminomethyl phenylalanine moiety to form a benzaldehyde derivative.3’ This oxidative degradant Is not detected In the neat solid drug stored under atmospheric oxygen. suggesting that oxygen is not involved In the reaction.3’ Instead, the decomposition of the heptapeptide may be due to a reaction with reducing sugar impurities in the man-nitol exclpient.3’ The proposed mechanism involves (1) formation of a Schiff base from the peptide primary amine reacting with the carbonyl of the aldehydic group on the reducing sugar, (2) tautomenlzation to a more stable configuration, conjugated with the phenyl group, and (3) hydrolytic cleavage of the new Schiff base to generate the observed aldehyde derivative.31 The first part of this proposed reaction, the formation of the Schiff base, pro-ceeds via a mechanism similar to the Maillard reaction, discussed below.
Mallard ReactionThe food industry has studied extensively the nonenzymatic browning offood due to the Malllard reaction, which results from reducing sugars reacting with either amino or free amine groups In proteins, leading to changes In both the chemical and physiological properties of the proteins (Scheme 4).3’ The first phase of the Mallard reaction involves a condensation reaction between the carbonyl of a reducing sugar and an amino group to form an N-substituted glycosylamine, which then converts to a Schiff base and a molecule of water.33

Subsequent cydlizatlon and isomerlzation (Amadori rear- rangement) resultIn derivatives which cause discoloration
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Scheme 5Oxidallan.

(nonenzymatic browning) of the forxnulation.n This type of covalent modification Is a problem in the solid state because the initial aminocarbonyl condensation reaction to form the Schiff base Is accelerated In the low moisture range.

Lyophilized human relaxin (Rlx) formulated with glucose was observed to degrade via the Mallard reaction to form adducts with glucose. The resulting adducts were shown by liquid chromatography/mass spectroscopy (LCIMS) to contain up to four glucose molecules covalently attached to Rlx25 With tryptic digestion, the Individualreaction sites on Rix were Identified as Including Lys,e, Lysag, and Argu17.25 The fourth reaction site was hypothesized to be either LYSA17 or Args13 on the T2-T7 fragment, which showed a broadened peak on the tryptic map, but positive Identification was not possible using mass spectroscopy.25 A review ofthe food literature prior to 1966 by Goldblith and Tannenbaum revealed that lysine loss In foods Is

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OH OH I
I+1130_

RCHCH + It’NB.2(Lys orAzg) - RCWc---H
untnb1e

R’

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CHrI4Hlti < C =CK

AmndsdRenl23gemcnt Enol

Scheme 4Mallard Reaction.

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,Y DH
Scheme 5fl Efiminallon.

primarily due to the Mallard reacdon.0 Lysine Is usually lost more rapidly than the other amino acids because of Its free &amlno group, which will react easily with the carbonyl of reducing sugars.n However, other residues, such as arginine, asparagine, and glutamhie, have been observed to react with reducing sugars also. fl-EliminationLyophilized bovine Insulin has been observed to degrade rapidly with Increased water content to produce both covalent and noncovalent aggregates after Incubation at 50 C at various relative humidities.36 Cos-tantino et al. hypothesized that the reducible covalent Interactions were due to thiol-catalyzed disulfide ex-change. They speculated that these free thiol groups resulted from the fl-elimination of Intact disulfide bonds In Insulin. The proposed mechanism for fl-elimination Involves hydroxide ion catalyzed cleavage of a carbon sulfur bond (cystelne) (Scheme 5A), resulting in two new residues, dehydroalanine and thlocystelne.36 Dehydroala-nine can then react with lysine to form a lysinoalanine cross-link, while thiocystelne can undergo further decom-position to form thiol-containing products, such as hydra-sulfide Ions (free thiols).36 A 5-fold increase In the level of free thiols was measured In lyophfllzed Insulin after Incubation at 50 C and 96% relative humidity.36 The free thlols formed during solid state aggregation were predomi-nantly low molecular weight (<3000 Da), perhaps hydro-sulfide Ions.36
Covalent Dimerizatlon and AggregationDimer-ization and aggregation differ from the other types of degradation reactions discussed In this review in that they are not the result of a single chemical change. In fact, many different types of chemical or physical changes can induce the formation of diners or aggregates. A brief description of chemically Induced dinierizatlon and aggregation Is included here because of its Importance In the pharma-ceutical Industry, and because much of the published literature on protein stability uses aggregate formation as

a key stability Indicator. Dimerization or aggregation via covalent cross-linkage can be categorized Into two types: reducible (via dlsulflde exchange) and norireducthle. Reducible Dimerizadon and Aggregadon-Lyophilized bovine serum albumin, ovalbumin, glucose oxidase, and fl-lactoglobulin were observed to form covalent Intermo-lecular disulfide linkages via a thlol-dlsulflde interchange reaction (Scheme 3D) Liu et al. postulated that the Intermolecular thiol-disulfide Interchange results from the Ionized thiol on one albumin molecule attacking the dis-ulfide linkage of another albumin molecule:

PJ-s- + P2-s-s-P2-- Pl-s-S-P2 + PZ-s-

where P1 and P2 are the first and second protein mol-ecules.37 Thiolate Ions rather than thiols are the reactive species In this reaction, since a decrease In the Initial solution pH resulted In a slower aggregation rate while an increase In pH led to a more rapid reaction.37 Free thiols are both necessary and sufficient for the moisture-Induced aggregation to occur, since S-alkylated BSA (no free sulfhydxyl groups) does not undergo aggregation? Moisture-Induced aggregation ofbovine Insulin occurred via both noncovalent and covalent Interactions. The cova-lent Interactions were reportedly due to intermolecular disulfide bonds as evidenced by the dissolution of 21% of the aggregates upon treatment with a reducing agent. 10 mM dithioerythrltol. and 1 mM EDTA to prevent Its autoxidation.m Costantino at al. postulated thatfl-elimina-tion produced free thiols which can subsequently catalyze the reshuffling of intact protein disulildes and lead to Intermolecular disulfide crossIInkage.3
Chemical Instability In the solid state can also occur after proteins first experience physical Instability, such as denaturation or unfolding. Lyophlllzed recombinant human keratinocyte growth factor (rhKGF) Is prone to aggregation at elevated temperatures.39 Its aggregation pathway Is proposed to proceed Initially with unfolding ofthe protein, leading to formation oflarge soluble aggregates, which can form disulfide bonds.-19 Finally, these precipitates are converted to scrambled disulfides and/or nondisulfide cross-linked oligomers. Recombinant human albumin (ri-IA) also undergoes Intermolecular thiol-disulfide Interchange.40 The unfolding or loss of tertiary structure In lyophilized rHA Is suspected to have initiated the covalent thiol-dlsulflde exchange.40 The covalent aggregation of ovalbu-mlii, glucose oxidase, and fl-lactoglobulin have also been linked to Initial protein physicalInstability, such as protein unfolding?
NonreducibleDirnerizatlon andAregadon-LyophllIzed human insulin can undergo covalent dlmerizatlon at the Asnp,zj site to form [Aspzi-Phea,] and [Asp-GlyA1j Insulin dirners.13 This dimerizatlon occurs after deanildation ofthe C-terminal AsnAzj forms a reactive cyclic anhydride Inter-mediate thatfurther reacts with the free N-terminal amine of another insulin molecule to yield dlmers.13 Lyophilized tumor necrosis factor (ThF) is also susceptible to the formation of cross-linked aggregates consisting of dimers, trlmers, and higher oligomers. which were nonreducible when treated with fl-mercaptoethanoL4’
Another protein that forms nonredudble dimers in the solid state Is recombinant bovine somatotropin (rbSt). When lyophlllzed rbSt was stored at 30 C and 96% RH, the rate ofloss ofmonomeric rbStwas greater than or equal to the rate ofloss In solution.12 Covalent dimers accounted for 80-90% of the total degradation products observed, with the remaining 20-10% attributed to deamldatlon/cleavage. The fractional amount of nonreducible dimers was only 23-26% in solution.12 Most of the covalent dimers were not reducible with -mercaptoethanol. Indicating that

the covalent bonds were not disulfide bonds.’? Hageman at al. hypothesized thatsuch covalent interaction could be due to the condensation of a lysine s-amine and a carbonyl side chain of either Asp or Giu.’2
A similar mechanism was proposed for the degradation oflyophilized ribonuclease A due to the formation ofsoluble and insoluble aggregates during storage at 45 C. These aggregates were not dissoclable using an anionic detergent (sodium dodecyl sulfate) and a reducing agent (2-mercap-toethanol), indicating that the aggregates were covalently attached but not through disulfide bonds.42 Originally, protein oxidation was believed to be the cause of the aggregation since exposure to oxygen and light increased the solid-state aggregation rate of ribonudease A.43 Upon further investigation. Townsend at al. postulated that these covalent linkages did not result from an oxidation reaction but from the lysine residues reacting with asparaglne or glutamine In a manner similar to the mechanism proposed by Hageman et aL forrbSt." The loss offree lysine residues in the Insoluble aggregates, as determined by amino acid analysis, Is consistent with this mechanism."
A loss of lysine and histidlne residues also was observed by Schwendenian at aL in studies of the moisture-induced aggregation of tetanus and diphtheria toxolds.45 In this case, however, aggregation was thought to be caused by formaldehyde-Induced cross-linking of the proteins, since the aggregates were not soluble under either reducing (10 mM dlthiothreltol, 1 mM EDTA) or denaturing (6 M urea) conditions, and since the toxoids had been treated with formaldehyde (°formalinlzed’) during production.45 The authors proposed a mechanism for the formation ofstable Intermolecular cross-links In this system and established methods for stabilizing the toxoid formulation against this reaction. Effective stabilizationmethods Included succinyl-atlon, treatment with sodium borohydride, and addition of
sorbltoL45

Factors Influencing Protein and Peptide Chemical Instability in Solids

The Importance of temperature, moisture, and formula-tion exciplents in determining the solid-state stability of small molecule drugs has been widely reported and accepted.4547 However, the effects of these factors on the solid state chemical stability of proteins and peptides are not as widely reported or understood. In addition to these factors, there Is growing literature thatsuggests that the hydrogen ion activity In the solid-state may have a signifi-cant Influence on peptlde and protein stability, paralleling the Influence ofpH In the solution state. The physicalstate of the solid (e.g., glassy vs rubbery) also has been shown to affect protein reactivity In the solid state. This section

reviews published reports ofthe effects of these factors on peptide and protein stability In the solid state: tempera-

ture, moisture, exciplents, hydrogen Ion activity, and the physical state of the solid.
Temperature-The exposure ofsolid protein and pep-tide formulations to elevated temperatures generally de-creases chemical stability by accelerating almost all chemi-cal degradation reactions. For example, the deamidation of the Asn-hexapeptlde Increases with Increasing temper-ature In the solid-state, with the formation of the cyclic Irnlde Intermediate becoming more favored over the direct hydrolysis of the Asn side chain.18 In general, the increase In degradation rates with temperature did not follow an Arrhenius relationship within the temperature range studied (40-70 °C).18 This suggests that acceleration of chemical reaction kinetics due to temperature Is notsolely responsible for the rate Increase.

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High-temperature inactivation of proteins often results from the destruction of disulfide bonds In cysthie via /3-elimination.’ Consistent with this, the aggregation of Insulin, in which /3-elimination is the first step, was observed to Increase with Increasing temperature. In-creases In temperature have been observed to Increase the rate of covalent cross-linking in the solid state for rbSt.12 bovine insulin,’ and tumor necrosis factor.4’ Most degra-dation reactions, such as dearnidatlon,18 peptide bond c1eavage,0 the Mallard reaction,n and oxidation" are accelerated by Increasing temperature.
The Increases in chemical instability with temperature may be due to Increased mobility In the system or lowering of the activation barrier for reaction. In particular, signifi-cant changes in mobility may occur when the temperature range ofinterest Includes the glass transition temperature (2’9) of an amorphous solid. In this case, changes In temperature affect not only the Intrinsic reactivity of the degrading species, as observed In the solution state, but also the medium In which the reaction occurs. The rela-tionship between the glass transition temperature (Td and reactivity Is discussed below, under "Physical State of the
Solid".

MoistureResidual moisture is often thought to be responsible for protein and peptide chemical Instability In the solid-state. In general, lyophilized protein formulations are more stable at lower water contents.2 For example, the rate of degradation of the Asp-hexapeptlde increases as the formulation vialmoisture content thcreases.n Likewise, the deamidatlon of the Asn-hexapeptlde Increases as water content Increases from 0.5% to 2%.18 However, further Increases in water content of up 3% do not significantly affect the rate of degradation.18
A similar effect of water on the deamidatfon of insulin was observed. The rate of degradation oflyophilized human Insulin increases steeply with water content at low levels of hydration, approaching apparent plateaus at water contents greater than The rate constants In the plateau region are comparable to those In solution.13 The rate of rbSt loss due to dearnidatlon and dimerization also increased with Increased water content12 Increasing the relative humidity from 75% to 96% doubled the rate ofrbSt loss,2 However, the fractional amount of nonreducible dimers formed remained constant at around 80% and was Independent of water content over the range studied.12 The covalent aggregation ofbovine serum albumin (BSA) In the solid state greatly depends on water content38 Freeze-dried BSA was more prone to aggregation In the moistened solid state than In solution and exhibited a maximum at a very low moisture level. The aggregation ofBSA in solution increased with increasing ratio ofwater to protein.38 Jordan et al. proposed that the Increase In aggregation rate Is related to the increase In water mobility with increasing ratios of water to protein.’ Lyophilized recombinant human lnterleukln-1 receptor antagonist (rhIL-Ira) formulated with 2% glyclne, 1% glycerol, and 10 citrate buffer at pH 6.5 exhibited maximum relative instability at a moisture content of around 0.8% (w/w) and the least stability at water contents less than 0.5% or around 3.2% (w/w).11 A similar bell-shaped relationship between covalent aggregation and water content has been observed for ovalbumin, glucose oxidase, and /3-lactoglo-bulin.37

A few other types of chemical Instabilities appear to exhibit this bell-shaped relationship between degradation kinetics and moisture content. A bell-shaped dependence on moisture content also was observed for the aggregation of rHA via thiol-disulfide exchange, with maximum ag-gregation corresponding to 47 g water/100 g dry protein (96% RI-I).40 The Mallard reactions usually have maximum

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rates in the low-moisture range.34 Insulin,59 casein,5’ and blood plasma proteins In "dry" systems containing glucose show a maximum in the range of 40-80% RH (10-20% water content).2
This bell-shaped relationship between water content and reaction rate Is often attributed to the effect of water on molecular mobility and reactant concentration. At low moisture levels, the Mallard reaction Is less rapid because the diffusion and mobility of reactants are restrIcted.LlZ As water content increases, molecular mobility increases, leading to enhanced reactant mobility which should facili-tate the reaction.37 At higher water contents, the reaction rates decrease due to dilution ofthe reactants by water.37.n Liu et al. observed that the addition of various types of polymers (carboxymethyl-cellulose, dextran, poly(ethylene glycol)) as dlluents also appeared to hinder the aggregation of BSA.37
While residual water can often Increase chemical deg-radation rates, under some conditions It can suppress a chemical reaction. Pikal et al. observed that the oxidation of a lyophilized hGH formulationwas affected by Increases In headspace oxygen at low moisture levels but not at higher moisture contents. This may be due to water’s possible antioxidant properties. Water may be either an antioxidant or an oxidant depending on the system.32 Generally, oxidation in foods reaches a maximum at low water content and decreases as water content increases.?

In addition to having an effect on solid-state degradation rates, water may also affect the reaction pathways. For example, the formation of the cyclic Irnide Intermediate during deanildatlon of the Asn-hexapeptide at pH 5 was favored over direct hydrolysis as water content Increased.18 The low moisture environment of the solid-state can generate product distributions which differ from those of the solution state.18 The main product In the degradation of the Asp-hexapeptlde in solids lyophilized from pH 3,5 and 5 solutions Is the cyclic Imide hexapeptide.0 In contrast, the dominant degradation product In solution at pH 3.5 is the tetrapeptide and at pH 5.0 Is the IsoAsp-hexapeptlde.24 Thus, the reduced moisture In the lyophi-lized solids appears to suppress the hydrolysis of the Asu-hexapeptide Intermediate to form the lsoAsp-hexapeptide and that ofthe Asp-Gly peptide bond to form the tetrapep-tide.

We have observed similar shifts In the product distribu-tion In our recentstudies ofAsn-hexapeptide deantidatlon In poly(vinyl pyrroildone) and poly(vinyl alcohol) matrices,2’ In addition, the order ofthe reaction with respect to water content differs In these two polymers: it Is first-order In poly(vinyl pyrrolidone), but second order In poly(vinyl alcohol).2’ Since It Is unlikely that the molecular deami-dationmechanism differs significantly In the two systems, the results suggest that the role of water extends beyond that of reactant and may involve plasticization, of the polymer matrices.
So, while the importance ofwater In chemical reactions Is well recognized, the exact relationship between solid-state chemical Instability and water content is often difficult to delineate and is dependent on the type of degradation reaction(s). This difficulty In determining the mechanism by which water affects the chemical stability of proteins and peptides in the solid state Is in part due to the various roles water can play In promoting chemical reactivity. Water can act as (1) a reactant, (2) a medium or solvent, and (3) a plasticizer to Increase molecular mobllity.2-47.54 Discriminating among these possible effects is difficult, particularly since more than one effect may be Important In a given system.
ExcipientsProteins are often formulated with excipi-ents, such as sugars and polymers, to protect them during

table 1Types of Polyalcabols Used as Pharmaceutical Exciplents

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freeze-drying and storage. Polymeric "exciplents" may also be used to form a matrix for controlled release. The types ofexciplents used and their effects on the chemicalstability of proteins and peptides are varied. For example, a wide range of exciplents, such as heparin. sulfated polysaccha-rides, anionic polymers, and citrate, decreased the rate of covalent aggregation In recombinant human keratlnocyte growth factor (rhKGF) at elevated temperatures.° On the other hand, while EDTA Inhibited disulfide formation In aggregates, It was not as effective In stabilizing rhKGF. In this section, three categories of exciplents will be discussed since they comprise the majority used In solid formulations: polyalcohols, polymers, and buffer salts. PolyalcoholsSugars and polyols are the most commonly used exciplents In lyophilized protein and peptide formula-tlor&5 Table 1 lists the different types ofpolyalcohols used as pharmaceutical exciplents. These sugars and polyols are often added to lyophilized formulations to protect and stabilize the proteins against decomposition during pro-cessing and storage. TNF formulated with dextrari, sucrose, trehalose, or 2-hydroxypropyl-fl-cyclodextrin, which are amorphous, formed fewer nonreducible dimers than TNF formulated without an exclplent.41 The covalent dimeriza-Lion of human Insulin was markedly decreased by Incor-poration into a glassy matrix of trehalose-56 The stability of lyophilized recombinant human lnterleukln-1 receptor antagonist (rhIL-lra) was greatly Improved by increasing the concentration ofsucrose In the formulation." Sucrose stabilized rhIL-Ira against chemical degradation better than trehalose." Examination of a series of polyalcohols In this system showed the following order of decreasing ability to stabilize the protein: sucrose > trehalose > sorbitol > no exciplent> maltose." In a recent study of Asp-Pro bond cleavage In Physalaemln and Hamburger peptides, stabilization was provided by glassy polyols in the order: sucrose> Ficoll (low mw) > Byco A, Ficoll (high mw) > dextran.51 The authors attributed the stabilizing ability of -the polyols to the density of the matrix, with greater packing density leading to Improved stability. Some of these exciplents, such as maltose In the rhlL-ira example above, may have a destabilizing effect. For example, the oxidative dearoldation of a cyclic heptapeptide was observed In formulations containing inannitol, but not In solution or In the neat solid (no exclpient).3’ The extent ofoxidative degradation Increases with increasingmarmitol content after 12 weeks ofstorage at 40 and 60 "C.3’ These researchers postulated that the cyclic heptapeptide was reacting with a reducing sugar Impurity In the mannitol.3’ Thus, the lower stability of the rHIL-ira maltose formula-tion may have been due to the rhIL-ira reacting with maltose, a reducing sugar.
Reducing sugars are known to react with proteins and peptides via the Maillard reaction (formation ofSchiffbase) as well as other side reactions Rapid covalent modi-fication of lyophilized human relaxin was observed In the presence of glucose.25 The glucose appeared to have reacted with lysine and arginine residues via the Mallard reaction to form covalent adducts.25 A significant amount ofserine cleavage from the C-terminal ofthe B-chain ofrelaxin was also identified. A high glucose content may be necessary

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for cleavage to occur, since the hydrolytic cleavage of relaxin stored in concentrated glucose solutions occurred to a lesser extent than In the solid state.?s Tn contrast, formulating relaxin with n9nreducg sugars such as mnannitoland trehalose produced stable, lyophilized for-
mulÆtlors.2

hi addition to oxidatloriand, deamidatlop products, human growth hormone formulated with actose produced an- addlttonal.prdduct not observed in either the freshly freeze-dried lactose formulation or ’In any of the other formulations studied. The researchers postulated that the product was due to an adduct of lactose and uGH, either the glycosylamirie or the corresponding Schiff base.4 As stated above, the use of reducing sugars In lyophilized formulations Is problematic due to their propensity to react with proteins via the Malliard reaction, especially at lysine and arginimie residues.
PolymersPolymeric exciplents may Influence the reac-tivity of peptides and proteins through direct chemical interactions or by altering the physical state (e.g., glassy vs rubbery) ofthe system. This-section addresses chemical interactions between polymeric exciplents and Incorporated proteins. The effects of polymers on the physical state of the matrix are discussed below.

Many ofthe published studies on the chemical effects of polymers on peptides and proteins Involve the lactidet glycolide copolymers. These polymers are of particular Interest because they are blocompatible and biodegradable and are therefore used commonly In Implantable devices. The stability of atriopeptin III (APIfl) encapsulated In poly(n,L-lactlde-co-glycollde) (PLG) microspheres was stud-led and compared to that of APIII alone at 40 "C.SB When microspheres; were shaken In neutral buffersolution, ANTI was completely degraded In 5 days. In neutral buffer alone. APIIT degraded with a half-life of 8,7 days.se After 8 days at 95% relative humidity, 20% ofAPUI In the microspheres had degraded.n Concentrated ANTIshowed no degradation after 2 weeks, and dilute APIII In water showed no loss after one monthm Different degradation peaks were ob-served for ANTI In microspheres and APIII alone.58 It was concluded that PLG catalyzes the degradation ofAPIU, and the mechanism of this degradation appears to be different from that seen for APIII alone In buffer.0 This suggests that the polymer environment has a significant effect on the chemical stability of this protein.
Park and co-workers also found this to be the case for the stability of carbonic anhydrase and bovine serum albumin during release from a polymer matrix Immersed In buffered medium at 37 "C.59 Carbonic anhydrase encap-sulated In poly(n,L-lactic add-co-glycolic acid) (PLGA) microspheres was released in an Inactive form.69 Severe hydrolysis of unreleased and released proteins was ob-served.° It was postulated that the hydrolysis was due to an acidified medium created by the degradation of the PLGA microspheres. The degradation of the PLGA ml-crospheres occurs via random chain scission of an ester linkage In the polymer backbone, which produces water soluble oligomers with carboxylic acid end groups that affect medium pH.59 As the microspheres degraded, the pH of the medium decreased from 7 to 3 over 40 daysP Park et aL also suggest that the protonated carboxylic acid end group of the lactic and glycolic acid fragments may react with the carbonyl group of the amide linkage In the proteins, leading to cleavage of the peptide backbone.59 So, In addition to having an environmental effect, polymers may also have a direct catalytic effect on the chemical stability of proteins In solids.
In contrast to these reports of a detrimental effect of lactide-glycolide polymers on protein stability, there have been several reports of successful protein formulation In

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these polymars.60 Cleland et aL describe the stability of recombinant human growth hormone (rhGH) during re-lease from PLGA microspheres into Isotonic buffer.50 Rates of oxidation, diketopiperazine formulation, and deamida-don In the microspheres were comparable to those in solution controls, and rates of aggregation were only slightly greater than In solutlon.GO The authors Infer that the pH within the microspheres is comparable to that In the surrounding buffer, and thatrapid buffer equilibration may prevent a pH decrease in this system.° In related studies, rhGH was stabilized In a PLGA mlcrosphere formulation by the formation of an Insoluble complex with zinc.8’ In vitro studies showed that microsphere encapsula-tion did not alter rhGH activity in this system, and In vivo studies in rats and monkeys showed sustained delivery of rhGH for up to one month.°’ The discrepancies in protein stability In PLGA systems suggest that factors other than the polymer choice will influence drugstability. These may Include the properties of the protein drug itself, other formulation variables, and the Interaction of the formula-tion with the release matrix (e.g., buffer, tissue). Limited Information Is available on the chemical effects of other polymeric exclpients on peptide and protein stability. Recent studies in our laboratories suggest that the Asn-hexapeptide forms a reversible complex with poly-(vinyl pyrrolidone) when stored In matrixes ofthis polymer at elevated temperature and low matrix moisture content.83 Formation of the complex results in a reduction In Asn-hexapeptide degradation to the cyclic Imide, but release of the Asn-hexapeptide from the complex requires more than I week in dialysis studies at neutral pH.63 The chemical nature of this complex is under investigation. Protein binding to dextrans In Iyophillzed proteins has also been suspected.84 After exposure to aqueous media for more than 2 weeks, recombinant bovine somatotropin (rbSt) and zinc insulin were released intact and active from microspheres composed of sucrose and poiy[I,3-bis(p-carboxyhydroxy)- hexane anhydride], which Is a very hydrophobic polymer.65 Ron at al. postulated that the hydrophobic polymer pre-vented the entry ofwater Into the rnicrospheres85 and thus protected rbSt from moisture induced decomposition, such as covalent dimerizatlon and deamldation.12
Buffer SaltsBuffer salts are often included In solid formulations of peptides and proteins in an attempt to control the hydrogen Ion activity, or the protonation state of the functional groups on the protein. In solution, buffer salts may have a catalytic effect on many protein degrada-tion reactions, apartfrom their effect on In this section, we review the limited evidence forspecific chemical effects of buffers In the solid state. The effects of solid-state hydrogen Ion activity on reactivity are discussed in the next
section.

There is some evidence that phosphate buffers have a catalytic effect on protein degradation. In a study on lyophilized RNase, Townsend and DeLuca prepared lyoph-ilized formulations from solutions containing varying con-centrations of sodium phosphate buffer.66 While a buffer-free formulation showed a 10% loss In activity over 120 days ofstorage at 45 C, a formulation lyophilized from a 0.2 M buffer solution showed a nearly 40% loss In activity during the same period.66 Similarly, Pikal et al. showed an effect ofsodium phosphate buffer concentration on the chemical degradation and aggregation oflyophilized human growth hormone.4 Mechanisms suggested for this effect Include the catalysis of oxidation reactions by heavy metal contaminants In the buffer salts,°° and the preferential crystallization of phosphate buffer components during freeze-drying, leading to pH shifts.4 Itshould be noted that sodium phosphate buffers are thought to be particularly prone to preferential crystallization, and often are avoided

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in lyophilized protein formulations for this reasonP Ac-etate buffers are also avoided In lyophilized formulations, since volatilization of acetate species during freeze-drying may alter the buffer composition In the solid.
Buffer species themselves may be chemically unstable In solid formulations, producing degradation products that Interact with peptides and proteins. Recent studies In our laboratories examined the deaniidatlon of the Asn-hexapep-tide in lyophilized matrixes containing PVP and Tris buffer.OB We observed the formation ofa new peak In HPLC chromatograms, which mass spectral analysis showed to be a formaldehyde adduct of the tyrosine residue on the aspartate or isoaspartate deamidation product. The pres-ence of formaldehyde was shown to be associated with the inclusion of Tris buffer In the formulation, since no formaldehyde was detected when Tris was omitted or replaced with other buffers.66 We hypothesize that degra-dation of Tris [trls(hydroxymethyl)amlnomethane] occurs on storage of this formulation, liberating formaldehyde, which then reacts with the tyrosine residue on the pep-

Hydrogen Ion ActivityIn the solution state, pH is used as a simple indicator of hydrogen ion activity. While pH Is not defined in the solid state, there Is a strong correlation between the chemical stability of proteins and peptides In the solid state and the pH of the solution prior to lyophllization. This value Is often termed the "apparent pH" of the solid and Is often given the abbreviated designa-tion, "p1-I", with the quotation marks signifying that the value Is apparent.
The deamidatlon rate of the Asn-hexapeptide increases as the solution pH prior to freeze-drying Increases from 5 to 8.1s The "pH" of the Asn-hexapeptide formulation solu-tion also appears to dictate the extent to which the peptide stability depends on other variables, such as moisture level and temperature."’ The deamidatlon oflyophilized human Insulin is also quite sensitive to "pH".13-14 Increases In the oxidation ofhGFL4’28 the fl-elimination ofcystelne in bovine Insulin,38 and the formation of nonreduclble dimers of tumor necrosis factor’ In the solid state all Increase with formulation "pH". Interestingly, while no dimers were observed at "pH" 4, the biological activity of TNF at "pH" 4 was significantly lower than at higher "pH’s".4’
The Increase in the rate of covalent dimerizatlon ofrbSt with Increasing "pH" is attributed to the deprotonatian of the lysine residues, which are more nucleophillc when deprotonated. Interestingly, the fractional amount of non-reducible dimers formed during rbSt degradation remained constant at around 80%, Independent of "pH" over the range studied (pH 5-10)."° Lyophilized recombinant human interleukin-1 receptor antagonist (rhIL-Ira) for-mulations containing citrate were less likely to form rioncovalent aggregates than those with phosphate." The researchers postulated that the destabilizing effect of phosphate buffer maybe due to the selective crystallization of the dibasic salt, which would expose the protein to a low

To avoid drastic p11 shifts, the weight ratio of buffer to other solutes should remain low,3 The addition of ionic agents such as NaCl should also be kept at a minimum or avoided. While NaCl does not affect "pH", the chemical stability ofhGH in freeze-dried formulations was decreased by the presence of NaCl,4 The researchers attributed the destabilizing effect of NaCl on hGH to a specific Ion effect, due primarily to the chloride ion.4 The effect of NaCl on hGH may also be due to a salt effect on "pK’.
The Physical State of the SolidThe state of a pharmaceutical solid depends on the temperature, compo-sition (drug and excipient), moisture, and on the time allotted to the experiment. While the effects of tempera-

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Lure, moisture, and formulation composition on chemical Instability were presented Independently above, their influences on the solid-state chemical stability of proteins and peptides are often synergistic. These variables com-bined determine the physical bulk state ofsolid protein and peptide formulations. The bulk state of a formulation (crystalline versus amorphous) has been linked to various cases of protein and peptide chemical Instability In the solld-state.413,18
CiystallineCrystalline solids possess long range mo-lecular order with the molecules structured In fixed geo-metric units. In contrast, amorphous solids have Irregular packing and lack long range order. For small molecule drugs In the solid-state, the crystalline drug is generally less prone to chemical decomposition than the amorphous form.1470 However, the crystalline state may not be more stable for protein and peptide formulations. Oilyai et at. found that the Asp-hexapeptlde was more stable In a lactose matrix (amorphous) than In mannitol (cxystalllne).n Interestingly, the lactose formulation had a greater affinity for water (a higher water content) than the mannitol formulation, yet the peptide was significantly more stable In the lactose matrix than In mannitol.’ Exciplents that were more hygroscopic were generally better atstabilizing rHA against aggregation, suggesting that the stabilization arises primarily from Interactions between the exciplent and water rather than between the exciplent and the protein.40
The stability of crystalline and amorphous Insulin (neat solid) was also Investigated at various water contents. For crystalline Insulin, dearnidatlon at the Asnii site increases rapidly with increases In water content.14 In contrast, the deamidation of amorphous insulin was nearly independent of moisture content (up to 15901).’ Crystalline human Insulin was less stable than amorphous insulin, indepen-dent of the water content over the range studied.14 Solid formulations which are at least partially amor-

phous appear to best protect proteins and peptides against Chemical instability.4 Lyophilized human growth hormone
(hGH) formulated in a partially amorphous exciptent system (glyclne:mannitol) was less susceptible to chemical degradation and aggregation than hGH formulated In either totally amorphous (dextran) or crystalline (mannitol) systems.4 So, while a partially amorphous system Is neces-sary to stabilize hGH, an amorphous system Is not a sufficient condition for stability.4 Pikal et at. postulated that a partially amorphous system allows molecular inter-action with the protein and/or acts as "sink" for residual
water.4

While residual moisture generally leads to chemical Instability, the relative stability of proteins and peptides In solid formulations cannot be Judged solely on the basis ofthe amount ofwater present, as seen In the above cases. The nature of the exciplent, such as its crystallinity and affinity for water, appears to be Important as well. AmorphousDepending on temperature, structure, and composition, amorphous solids such as polymers exhibit widely different physical and mechanical properties.7’ At low temperature and moisture, amorphous solids are brittle, hard, and highly viscous, and are said to be In the "glassy" state. In the glassy state, molecular motion Is largely restricted to vibrations and short-range rotational motion.7’ As the temperature and/or moisture content increases, the solids undergo a transition from a glassy state to the so-called "rubbery" state, which Is characterized by decreased viscosity and increased elasticity. The glass transition temperature (T3) is defined as the temperature atwhich the solid softens because of the onset oflong-range coordinated molecular motion.7’ The T. varies greatly among different amorphous pharmaceutical solids and Is

dictated by such factors as composition and moisture content, since water may act as a plasticizer to increase bulk mobility and decrease To.
Researchers have speculated that chemical reactions which involve molecular mobility, such as bimolecular reactions, are unlikely to occur In the glassy state due to the limited mobility. As the solid system becomes more rubbery (T> To and mobility Increases, the reaction rates would then greatly Increase.Th Lyophilized rhIL-Ira for-mulations with T5’s ranging from 20 to 56 "C were stored for several weeks at temperatures above and below their 2’s.73 The decomposition ofrhIL-ira, both via deamidation and aggregation, was greatly accelerated at temperatures above T. (rubbejystate).ri However, the rate of degradation also increased in some formulations whose 7’s were above

the storage temperature (glassy state).ri
Other Instances of protein and peptide degradation In the glassy state have been documented. The aggregation of hGH has been found to occur in formulations stored at temperatures well below their glass transition tempera-ture.4 The dimerlzatlon and deaxnldatlon of Insulin have been observed In the glassy state;13 however, the actual formulation T. was not measured In this study. Recently, the fission of the Asp-Pro bond In Physalaemln and Hamburger peptides has been detected In water soluble glasses (e.g., dextrose, Ficoll, sucrose) below their 7n Thus, while proteins and peptides are generally more stable In the glassy state, chemical degradation reactions can still occur. Although mobility is limited In the glassy state, glassy pharmaceutical solids may experience significant molecular mobility at temperatures up to 50 "C below their glass transition temperature.74 Furthermore, significant rotational and translational mobility was shown to exist for water in solid poly(vinyl pyrrolidone), even at very low water contents (glassy state).75
The Increase In reactivity with water content Is often attributed to water’s ability to plasticize or Increase the molecular mobility of the bulk structure and thus the reactants. The decomposition of a freeze-dried monoclonal antibodyymca conjugate has been correlated with formu-lation 2 However, since water activity and content were not kept constant, other factors which may contribute to this Instability cannot be ruled out. In contrast, the degradation of aspartame in the solid-state correlates better with water activity and content than with T5.77 In recent studies of Asn-hexapeptlde dearoldation In poly-(vinyl pyrrolidone) matrices, we have attempted to dis-criminate between water’s effects on T. (i.e., as a plasti-cizer) and Its solvent effects by adding glycerol to the matrix as a secondary plasticizer. This enabled us to generate systems with constant T5 and varying water activity, or with constant water activity and varying 7. Interestingly, the deamidatlon rate showed a single log-linear correlationwith T for matrices In the rubbery state, while In the glassy state this correlation resulted in multiple parallel lines with Intercepts dependent onmatrix water activity. These results suggest that forthis system, water acts primarily as a plasticizer in the rubbery state, but acts both as a plasticizer and a solvent or reaction medium in the glassy state. Taken together, these conflict-ing reports on water’s role as a plasticizer demonstrate the complexity of these systems and the attendant difficulty in gaining a mechanistic understanding of the chemical reactivity of incorporated proteins and peptides.

Summary and Commentary

The results discussed above highlight the difficulty In determining the mechanisms ofprotein chemical Instability In the solid-state. Protein decomposition in the solid state

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can occur through multiple degradation pathways, Involv-ing both physical and chemical Instabilities, as observed for rbSt and insulin. Since chemicalInstability can also lead
to physical Instability and vice versa, It can be difficult to determine the mechanism of chemical degradation In proteins. Furthermore, non-peptide/protein components of these solid-state formulations may have multiple effects. Residual moisture and polymeric exciplents, for example, may influence matrix mobility CT5), may participate directly or Indirectly In degradation reactions, and may Influence the "pH" or other properties of the reaction medium. Multiple effects caused by a single additive make mecha-nistic definition difficult.
Consideration of the available literature In this area suggests several avenues for additional research. Most notably, It is clear that the determinants of protein and peptide reactivity In the solid-state have not been fully characterized. While pH, temperature, and buffer composi-tion are known to be critical In solution, different or additional factors may be Important In the solid-state. These may Include mobility In the matrix, hydrogen ion activity, and protein secondary and tertiary structure. Additional research relating to each of these factors Is warranted.

For example, while mobility In a solid polymer matrix is often characterized using the glass transition tempera-ture (T5), this measurement describes the mobility of the matrix as a whole and not Its specific components. Ulti-mately, the degradation of peptides and proteins In these systems Is more likely to depend on the mobility ofthe drug molecules themselves or of specific amino add residues within them, rather than on the "bulk" mobility described by 7, particularly when the drug loading is low. Recent research has begun to address this question.79-75 but continued effort will be required to establish a measure of solid-state mobility that Is predictive of reactivity. In addition, mobility Is likely to be more Important for bimolecular reactions involving large molecules (e.g., pro-tein aggregation) than for unimolecular reactions (e.g., succinimide formation) or bimolecular reactions Involving relatively small, mobile reactants (e.g., oxidation). An understanding of the factors that produce a "mobility-limited reaction" In the solid state would aid hi formulation, since stabilization strategies that reduce mobility (e.g., Inclusion of glass-forming excipients) are most likely to be effective for these reactions. Reaction mechanism and reactant molecular weight are likely to be among these factors.

Hydrogen Ion activity (I.e., pH) is important In the solution state degradation ofmany peptides and proteins, but ltsrole In the solid-state remains undear. At present, there is no convenient measure ofhydrogen Ion activity In these solid systems, so examination ofIts effects has relied on correlation with "effective" pH. These studies have suggested that the "pH" dependency in the solid state parallels its solution state counterpart. However, It is reasonable to expect that a reaction that Is pH-driven In solution may be controlled by other factors, such as mobility, in a glassy solid. Examination ofthe dependence ofsolid-state "pH" rate proffles on other matrix properties would be useful, as would the development of methods to measure hydrogen ion activity In solid formulations. Finally, the effects of protein secondary and tertiary structure on reactivity and reaction mechanism should be examined. In solution, secondary structure has been shown to affect deamidatlon rate.50 and protein aggregation in the solid state has been attributed to adsorption-induced unfolding.52 In addition, a recent study has demonstrated the utility of infrared spectroscopy to determine protein secondary structure In PLGA matrixes.8’ However, there

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is a lack of Information on the effects of protein structure on chemical reactivity In solids. Such Information is Important not only for chemical routes of protein degrada-tion, but also because physical modes of degradation, such as aggregation, are often associated with chemical changes. Information on the Interaction of protein structural do-mains with matrix properties (such as mobility and water content) to produce degradation would help to Identify labile residues In solid protein formulations a priori. Stabilization strategies could then be directed In a rational way toward the modification of protein structure and/or
formulation variables.

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(54) Title; STABLE LYOPHILIZED PHARMACEUTICAL SUBSTANCES FROM MONOCLONAL OR POLYCLONAL ANTIBODIES

(54) Bezelchnung: STABILE LYOPHILISIERTE PHARMAZEUTISCHE ZUBEREITIJNGEN VON MONO- ODER POLYKLONALEN
ANTIKORPERN

(57) Abstract

Disclosed are lyophilized pharmaceutical substances from monoclonal or polyclonal antibodies, containing a sugar or amino sugar well as a surfactant as a stabilizing agent. Also disclosed are the method of preparing said stable lyophilizates as well as the use of a sui or amino sugar, an amino acid and a surfactant as stabilizers for therapeutic and diagnostic agents containing antibodies.

(57) Zusammenfassung

Die Erfindung betriffi lyophilisierte pharmazeutlsche Zubereintungen von mono- oder polyklonalen Antikorpern, die einen Zucker oder Aminozucker, elne Aminosäure und cia ’I’ensid ala Stabilisierungsmittcl enthalten. Aullerdem betrifft die Erfindung elm Verfahrcn zur Herstellung dieser stabilen Lyophilisate sowle die Verwendung elnes Zuckers oder Aminozuckers, einer Amlnosäure und eines Tensids ala Stabilisatoren von antikorperhaltigen therapeutischen oder diagnostischen Mltteln.

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TECHNICAL NOTE

Freeze-Dried Formulation for Direct 99mTcLabeling ior-egf/6
MAb: Additives, Biodistribution, and Stability

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ABSTRACT. Monoclonal antibodies (MAbs) have been useful for immunoscintigraphic applications in clinical diagnosis since they were introduced in nuclear medicine practice. The MAb ior egf/r3 developed at the Center of Molecular Immunology (Havana, Cuba) is a murine antibody that recognizes the human epidermal growth factor receptor (EGF-R) and has been used widely in the radioimmunodiagnosis of tumors of epithelial origin. Based on the direct Schwarz method, the present report describes the preparation of a freeze-dried formulation for radiolabeling the MAb ior egf/r3 with 11Tc for immunoscintigraphic applica-tions. Radiolabeling efficiency, effects on immunoreactivity, biodistribution, pharmacokinetic, and stability of the formulation are reported. The study demonstrated that the freeze-dried formulation can be labeled with at high yield. The resulting mTc.labeled ior egf/r3 MAb can be used to visualize in vivo human tumors of epithelial origin by immunoscintigraphy studies. The kit does not need any other addition or purification at the time of tagging other than the requisite amount of pertechnetate (40-50 mCi). Because the contents of the kit are lyophilized, no special storage or transportation is required. NUCL MED BIOL 266 717-723, 1999. ' 1999 Elsevier Science Inc. All rights reserved.

KEY WORDS. Monoclonal antibodies, Radiolabeling. Technetium-99m, Biodistrihution, Freeze-dried

INTRODUCTION

The development of monoclonal antibodies (MAbs) of high affinity and high specificity for tumors has generated widespread interest in radiolabeled MAbs for radioimmunodiagnosis. Many reports on clinical investigations using radiolabeled antibodies for diagnosis of malignant neoplasms have been published (1. 5, 14, 30). Although the feasibility for imaging has been established with MAbs labeled

to long-life radionuclides such as 1311 (5) and t’ln (8, 26), improved imaging quality had been obtained by using mTc (13). In these applications 99’Tc has almost replaced other radionuclides due to its nuclear properties and its availability from a generator (25). The direct method for radiolabeling a reduced MAb with 99mTc in the presence of methylene diphosphonate (MDP), developed by Schwarz and Steinstrasser (35) and Mather and Ellison (22), has been proved to be a good procedure to obtain high labeling efficiency and a stable labeled MAb. The ’Tc-MDP method uses reduction of disulfide bridges of the molecule by adding an excess of the reducing agent, 2-mercaptoethanol (2-ME). After purification to eliminate the excess of 2 -ME the reduced antibody is labeled with technetium via Sri21 reduction of pertechnetate, using MDP as a weak competing ligand. Direct methods are efficient and adaptable to a kit type ofradiolabeling procedure. Nevertheless, the use of a reducing agents may affect immunoreactivity of the reduced

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antibody and the labeled antibody could be unstable. These characteristics have to be evaluated.
The human epidermal growth factor receptor (EGF-R) is a transmembrane glycoprotein of 1,186 amino acids with a molecular weight of 170 kDa, containing an extracellular EOF-binding do-main, a transmembrane domain, and a cytoplasmic domain, which has tyrosine kinase activity and undergoes autophosphorylation upon ligand binding (3, 4). EGF-R overexpression has been found in a variety of malignant epithelial tumors arising in different organs such as breast (28, 31), bladder (27), lung (8, 39), and gliomas (37). The ior egf/r3 MAb developed at the Center of Molecular Immunology (Havana, Cuba) is a murine antibody that recognizes the EGF-R. Its properties have been described previously (11, 12). When bound to the membrane-receptor complex it becomes inter-nalized with the receptor, causing down-regulation of the EGF-R without stimulation of tyrosine kinase activity. These characteris-tics have been used widely in the radioimmunodiagnosis of tumors of epithelial origin (32).
The present report describes the preparation of a freeze-dried formulation for radiolabeling the ior egf/r3 MAb with 99"Tc for immunoscintigraphic applications. Radiolabeling efficiency, effects on immunoreactivity, biodistnbution, and stability of the formula-tion are reported.

MATERIALS AND METHODS

Antibody

The MAb br-c isFrighly specific-inurine IgU2a isotype thatL xecognize&the--EOF-R It was obtained and produced in the Center

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of Molecular Immunology (Havana, Cuba) by standard hybridoma techniques as described previously (11).

Antibody Reduction

The MAb was reduced by reaction with a molar excess of 2-ME 2,000:1 (2-ME:MAh) (23, 24).

Freeze-Dried Kit Formulation

Aliquots containing 3 mg (1.5 mL) of the reduced antibody, 15 mg of different additives, and 150 p1 of the MDP bone scanning kit (Amerscan, Amersham, UK) were mixed and frozen with liquid nitrogen (23, 24). Ad1tiyes -such w
sucrose, mannitol, andinosittzlwere used -at l%:Thenrhefrozen :products were4yophilized for 24 h;sealed undevacuuman&stored --a4Corth1 use.:Freeze-dried formulations were challenged under two different conditions designed to test their stability in time. Freeze-dried samples were stored for a week at 37°C and 24 h at 45°C. Labeling efficiency and antibody immunoreactivity were tested for all samples.

Suiphydryl Groups

The number of free sulphydryl groups was determined with Ellman’s reagent (10) and a micromethod developed in our laboratory and described previously (17, 18). Results were expressed as sulphydryl groups per antibody molecule.

Radiolabeling

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injected intravenously (Lv.) via lateral tail vein with a total volume of 0.3 mL. After injection groups of rats (n = 3) were perfused immediately and blood samples were taken at time intervals of 1, 4, 8, 16, 24, and 48 h. The liver, kidneys, lungs, spleen, and intestine were excised, rinsed of residual blood, and weighed. Samples were counted for radioactivity in an automatic gamma-counter (Wizard, model 1470) with window setting set to detect the 140-keV photopeak of Organ activity was expressed as percent of injected doses per gram of tissue (%JD/g).

Pharmacokinetics in Rats

Thirty female Wistar rats were administered I mg/200 pCi dose in a total volume of 0.3 mL by i.v. bolus infusion via lateral tail vein. Following i.v. injection, 1-mL blood samples were collected in all groups of rats (n = 3) at timed intervals. The total counts in serum samples were then converted to activity concentration in Ci/mL. For model-dependent analyses, the best estimates were obtained by fitting a biexponential model to the serum activity versus time data. The first and second phase half-life (T112) were calculated from standard pharmacokinetic equations using first-order rate constants.

Stability of the Freeze-Dried Formulation

Radiolabeling of the ior-egf/r3 MAb using the freeze-dried formu-lation was carried out at various periods (up to 1 year) after its preparation (three lots). The volume (1-2 mL) and radioactivity (40 mCi) of pertechnetate were kept constant in each labeling. The labeling efficiency, radiocolloids, and antibody immunoreactivity were calculated each time to evaluate the stability of the freeze-dried formulation.

Vials of iyiphilized antibody were reconstituted with 4-2- mL
(40-50 TmGI) oiperteehnetare RESULTS Suiphydryl Groups

Quality Control

The labeling efficiency was determined using ascending paper chromatography on Whatman 3MM paper and 0.9% saline and
methyl ethyl ketone (MEK) (23). Radiocolloids were determined
using human serum albumin (HSA) impregnated instant thin layer chromatography-silica gel (ITLC-SG, Gelman Sciences) and NH40H:ethanol:water (1:2:5) as the mobile phase to separate radiocolloids that remained at the origin (38).

Antibody lmmunoreactivity

The effect of the reducing process on the immunoreactivity of the antibody was measured by radiorcccptor assay (15. 21, 24) using human placental membrane extract, which has a high expression of EOF-R.

The immunoreactive fraction of the labeled antibody using the Lindmo method was also determined (20). H-125 human lung adenocarcinoma cells (American Type Culture Collection, Rock-ville, MD) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics at 37°C and 5%
CO2.

Biodistribution Studies in Rats

Biodistribution studies in rats were performed by injection of 9mTc labeled MAb ior-egf/r3 (1 mg/200 p.Ci). Fifteen rats were

The average number of -SH groups obtained was 6.65 – 0.69 and 6.28 – 0.48 groups per molecule of antibody for reduced antibody and the freeze-dried antibody without additives, respectively.

Labeling

Both the reduced MAb and the freeze-dried antibody showed good results with mean of 98.7% and 98.5%, respectively.

Antibody Immunoreactivity

Figure 1 shows results obtained for immunoreactivity of the reduced antibody and the freeze-dried antibody. Concentrations of antibody that caused 50% inhibition were compared: 19.63 p.gJmL for nonreduced control antibody and 19.27 and 21.07 p.g/mL for reduced MAb and freeze-dried antibody.

Freeze-Dried Formulations with Additives

LABELING EFFIcIENCY. Table I shows the labeling efficiency results. Formulations with mannitol and sorbitol showed labeling efficiency of 90.8 – 0.79% and 92.5 – 2.11%, respectively. Tc-MDP was the major impurity for these two formulations (8.9% and 7.3%, respectively). Formulations with trehalose, glucose, sucrose, and inositol showed labeling efficiency greater than 97.5%.

ANTIBODY IMMUNOREACTIVITY. Antibodies from formulations

and the native control antibody were able to compete completely

Freeze-Dried Formulation for Direct mTcLabeling ior-egf/r3 MAID

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12000

8000

[S]

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c(ior-egf76) ji.tg/mL

- ’-control mAb -a--reduced mAb -I-- lyophilized mAb

with radioiodinated EGF for the binding to EGF-R at similar antibody concentrations (Fig. 2).

STABILITY OF THE FREEZE-DRIED FORMULATIONS. Stabilizing effect
on the labeling efficiency was observed in freeze-dried samples in the presence of trehalose and glucose (Table 2) In contrast, sorhitol and inositol provided substantially lower protection. Radio receptor assay did not show any measurable loss in imniunoreactivity of the antibody for all formulations (data not shown).

Freeze-Dried Formulation with Glucose

RADIOLABELING. Labeling efficiency of 98.13 – 0.19% (media – SD) was obtained for four lots of the freeze-dried formulations.

ANTIBODY IMMUNOREACTIVITY. The results of the Lindmo anti-body immunoreactive fractions are shown in Figure 3. It was found to he 0.95.

RADIOCOLLOIDS DETERMINATION. Only 0.86 – 0.39% of the radioactivity corresponded to radiocolloids for the freeze-dried formulation,

TABLE I. Labeling efficiency for MM, ior egf/r3 Freeze-Dried With Different Additives Measured by Paper Chromatography Using Saline and Acetone as Mobile Phase

Additives Saline Acetone

PBS 99.43 0.35 99.60 0.14
Trehalose 99.62 0.22 99.81 0.03
Glucose 98.47 0.45 99.55 0.21
Sorbitol 92.42 2.11 99.63 0.28
Sucrose 99.47 0.28 99.65 0.07
Mannitol 90.65 0.79 99.62 0.14
Inositol 97.50 1.43 98.72 1.82

MAb = monoclonal antibody; PBS = phosphate-buffered saline.
Each value is the mean – SD of triplicate results.

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FIG. 1. Antibody immunoreac-
tivity measured by radio receptor
assay as described in Materials
and Methods. Reduced mono-
clonal antibody (MAb) and ly-
ophilized MAID were compared
to the nonreduced control MAID
(native) for their ability to com-
pete with the radioiodlnated epi-
dermal growth factor.

BIODISTRIBUTION STUDIES IN RATS. Biodistribution studies in rats are shown in Table 3. Among all organs, significant accumulation of the radiolabeled was found only in the kidneys (2.06 – 0.61%ID/g, mean – SD) at 1 h postadministration. This value remained high during 48 h postinjection. Localization of the radioactivity in the liver following injection of 9°"Tc4aheled ior-egf/r3 MAb was high at 4 h, but decreased to 0.44 – 0.10 and 0.19 – 0.02%!D/g at 24 and 48 h, respectively. A good clearance was also seen from the lungs, 1.31 – 0.30%ID/g at 4 h, down to 0.33 – 0.021)uID/g at 48 h.

PHARMACOKINETIC STUDIES. The radioactivity content of rats’ serum expressed as concentration of activity per milliliter of serum was plotted against the time of blood drawing in a linear plot. Serum time-activity curves were best fit to a biexponential equation with a distribution half-life (t112) of 1.14 – 0.11 h (mean – SD, n = 3) and elimination half-life (t3) of 24.41 – 2.23 h, respectively.

STABILITY OF THE FREEZE-DRIED FORMULATION AT 4°C. The effect of the duration of storage of the freeze-dried formulation on the quality of the radiolabeled antibody was examined to evaluate the shelf life of the formulation. We labeled the formulation with

99m-Tc after storing it for different time intervals up to a period of 1 year (Table 4). These data show that even when storing the formulation for 1 year the labeling efficiency was higher than 90%. The freeze-dried formulation without additives as preservative showed poor stability. Labeling efficiency was 80% when samples were stored 3 months at 4°C.

DISCUSSION

The ior egf/r3 MAb has been radiolabeled with)9mTc using the technology developed by Schwarz and Steinstrasser (35) and mod-ified by Mather and Ellison (23) and has been used successfully in the diagnosis of tumor of epithelial origin (24, 32). Nevertheless, the reduced MAb is unstable in solution and must be stored at -20°C to protect the -SH groups. Freeze drying, or lyophilization, is commonly used in the manufacture of protein products that are

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FIG. 2. Antibody immunoreac-
tivity measured by radio receptor
assay as described in Materials
and Methods. Reduced mono-

clonal antibodies (MAbs) feeze-
dried with different additives
were compared to the nonre-
duced control antibody (native)
for their ability to compete with
the radioiodinated epidermal
growth factor.

insufficiently stable for distribution and use in aqueous solution. These results showed that the freeze-dried process has only very little effect on the number of -SH groups generated in the MAb. Nevertheless, the freeze-dried MAb was unstable at 4°C, showing a labeling efficiency of 80% when stored during 3 months. Even when the formulation survives freeze drying without significant damage, degradation occurred during storage of the dried product.
Given the historical use of sugar as lyoprotectants and stability enhancers (2, 6, 7), we decided to include several carbohydrates as additives in the freeze-dried formulation. Additives in the formula-tions can have a major impact on the design of the freeze-drying process and can largely determine the in-process degradation of the protein and the stability of the dried product. Sugars such as trehalose (2. 9, 16, 33, 34), glucose (9), sucrose (9, 33), and mannitol (19, 29) are commonly used as lyoprotectants. All of the sugars tested protected the immunoreactivity of the reduced MAb; the labeling efficiency obtained was higher than 98%. Only mannitol and sorhitol did not protect the reduced MAb when radiolabeling with 99"Tc was carried our; labeling efficiencies of

TABLE 2. Stability of the Freeze-Dried Formulations When Samples Were Incubated at 37°C During I Week and at 45°C During 24 h

Additives

1 week at 37°C

Saline Acetone

24 h at 45°C

Saline Acetone

PBS 0.6 3.3 2.7 5.1 Trehalose 98.5 99.0 99.2 99.4 Glucose 63.0 63.8 91.5 93.4 Sorbitol 7.5 13.4 41.5 50.3 Sucrose 0.3 3.8 27.2 28.0 Mannitol 12.2 22.2 39.0 53.0 Inositol 3.4 6.3 1.0 4.2

PBS = phosphate-buffered saline.
Each value is the mean of duplicate results.

A. A. M. Morales et at.

90.65% and 92.42% were obtained, respectively. Mannitol as a bulking agent is one of the established excipients used to stabilize freeze-dried proteins. Nevertheless, a stabilizing effect of 50% has been reported for mannitol compared with other sugars (29). This poor effect may have its origin in the tendency of mannitol to crystallize during freeze drying, thereby removing most of the mannitol from the protein phase and reducing its stabilizing effect. However, because crystallization was not examined, no definitive statement can be made.

The stability of the freeze-dried formulations was studied at high temperatures. The best results were obtained for trehalose and glucose as additives. The stabilizing effect of trehalose was observed even when samples were stored for a week at 3 7°C. Of the other sugars tested, only the monosaccharide glucose, which is often used as a cheap protectant during the freeze-drying of proteins, provided protection over -SI-I groups in the reduced MAb. Although the protective efficiency of glucose did not reach that of trehalose, the use of glucose as an additive is a good selection for freeze-dried formulation. Therefore, it is necessary consider whether it is better, for a particular purpose, to choose the expensive, nontraditional, but well-defined trehalose or to use the cheaper, traditional, and good glucose excipient for freeze-dried formulation.
We selected glucose and four lots were prepared to study the pharmaceutical quality of the freeze-dried formulation. Radiolabel-ing efficiency was 98.13 – 0.19% and radiocolloid determination showed less than 1%. This control is especially important for freeze-dried formulations because the lyophilization process could induce colloid formation caused by changes in the oxidation state of
Sn2

A simplified kit for instant preparation of mTchuman IgO has been described with good stability at 4°C (36). Nevertheless, our freeze-dried reduced MAb was not stable at 4°C and labeling efficiency was 80% when freeze-dried MAbs were stored 3 months. Glucose as additive improved stability considerably; even after 1 year, labeling efficiency was higher than 90%.

Freeze-Dried Formulation for Direct mTc,Labeling ior-egf/r3 MAb 721

FIG. 3. linmunoreactive frac.
tion (r = 0.95) by Lindmo
method. Total/Bound = total ap..
plied radioactivity over specific
binding of the ’Tc-labeled
monoclonal antibody (MAb) ior
egflr3 versus the inverse cell
concentrations showed good cor-
relation coefficient (0.9922).

FIG. 4. Pharmacokinetic studies
in rats. Serum clearance follow-
ing Tc-labeled monoclonal
antibody (MAb) ior egflr3 injec-
tion. Values plotted represent
the mean of three animal data
points per group.

TABLE 3. Normal Organ Biodistribution of ’Tc-Labeled MM, ior egf/r3 (Freeze-Dried Formulation With Glucose as Additives) in Rats
% Wig tissue

Organ I 4h 8h 16h 24h 48h

Liver 0.56 0.13 1.12 0.10 0.51 0.02 0.40 0.02 0.44 0.10 0.19 0.02
Kidneys 2.06 0.61 2.84 0.25 2.63 0.18 3.19 0.41 3.86 0.16 2.29 0.41
Lungs 0.77 0.03 1.31 0.30 0.84 0.07 0.66 0.11 0.47 0.04 0.33 0.02
Intestines 0.31 0.06 0.56 0.13 0.36 0.05 0.34 0.03 0.24 0.03 0.23 0.07
Stomach 0.17 0.03 0.25 0.04 0.17 0.03 0.24 0.04 0.20 0.03 0.20 0.03
Pancreas 0.13 0.02 0.20 0.06 0.23 0.03 0.17 0.02 0.16 0.02 0.16 0.00
Brain 0.02 0.00 0.06 0.01 0.02 0.00 0.03 0.00 0.03 0.00 0.03 0.00
Spleen 0.16 0.03 0.44 0.06 0.35 0.03 0.32 0.03 0.20 0.03 0.17 0,03

MAb = monoclonal antibody.
Results are expressed as the percentage of injected dose per gram of tissue (% ID/g) as described in Materals and Methods.

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TABLE 4. Stability of the Freeze-Dried Formulation With Glucose as Additives After Storing the Kit for Different Periods

Time Freeze-dried (PBS) Freeze-dried Glucose Labeling efficiency Labeling efficiency Colloids Colloids

(Months) Saline Acetone (%) Saline Acetone (%)

0 98.95 0.21 99.05 0.28 0.95 0.45 98.13 0.19 99.12 0.59 0.86 0.39
3 80.50 2.26 80.92 3.05 1.05 0.39 97.87 0.93 99.53 0.50 0.74 0.47
6 52.75 1.25 53.25 0.90 0.92 0.52 98.12 1.15 99.40 0.41 0.92 0.21
8 94.32 0.12 99.00 0.16 0.63 0.15
10 92.55 3.14 99.35 0.21 0.68 0.16
12 92.94 5.33 99.03 0.32 0.95 0.48

PBS phosphate-buffered saline.
The freez-dried formulation was labeled with "Tc and the labeling efficiency was measured by paper chromatography. Results are expressed as mean SD.

We investigated the biodistrihution and elimination of the
9’Tc4abcled MAb following a single i.v. bolus infusion of the
freeze-dried formulation in rats. The 9’"Tc-labeled MAb cleared

rapidly from circulation, decreasing nonspecific uptake of radioac-
tivity in normal tissues, other than the liver, lungs, and kidneys,
which retain the radiopharmaceutical. The significant amount of radioactivity in kidneys during the first 24 h also suggests radio-pharmaceutical clearance via the urinary bladder route. The mTc labeled ior egf/r3 MAb was cleared from circulation with biexpo-nential kinetics. An initial phase, or alpha phase, with a half-life of 1.14 h, representing in part the distribution of the antibody between the vascular and extravascular compartment, and a second phase, or beta phase of 24.41 h, representing the catabolism of the antibody fully equilibrated between the two compartments.
The study demonstrated that the freeze-dried formulation could
he labeled with mTc and that the resulting samTc..Iabeled ior
egf/r3 MAb can be used to visualize in vivo human tumors of epithelial origin by inimunoscintigraphy studies. The kit does not need any other addition or purification at the time oftagging except the addition of a requisite amount of pertechnetate (40-50 mCi). Because the contents of the kit are lyophilized, no special storage or transportation is required.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO DIVISION DE NUEVAS CREACIONES

Abogado
EMILIO FERRERO
(Apoderado)
MERCK PATENT GMBH.,

ASUNTO Radicaciôn 04-57564
TrÆmite 002
Evento 001
Actuación 430

Oficio 107234
Folios 05

Patente de Invención Ej Patente de Modelo de Utilidad

Via Nacional E

Via PCT (fase nacional). Cap] Cap. II

No. Sol. Internacional PCT/EP02/1 3223

Fecha de Presentaciôn Internal. 25/11/2002

FM-

Como resultado del examen de fondo, realizado con fundamento en el artIculo 45 de a Decision 486 de la Comisiôn de la Comunidad Andina, se le comunica:

1. Documentos estudiados

Descripción: Folios: 40 a 66 como se radicO inicialmente

Reivindicaciones: Folios: 79 a 82 como se radicO inicialmente
Folios: 131 a 132 como se modificô el 23/01/08

Fiauras: Folios: 77 a 81 como se radicO inicialmente

Argumentos del solicitante: Folios: 120 a 129

Prioridad: Folios: I
Fecha: 01 de Noviembre de 2002
Pals: GB
NUmero: 0225520.6

2. Objeto de la invención

Titulo de la solicitud (folio 1): "Preparación liofilizada que contiene un anticuerpo contra el receptor EGF".

La presente solicitud se refiere a una preparaciôn farmacØutica liofilizada de anticuerpos que comprende un azUcar o un amino azücar, un aminoÆcidoy
un surfactante.

El problema planteado en esta solicitud es la necesidad de una preparaciôn para estabilizar anticuerpos.

Para solucionar este problema tØcnico Ia solicitud en estudio proporciona una preparación farmacØutica liofilizada de anticuerpos mono o policlonales que comprende un azücar o un amino azUcar, un aminoÆcidoy un surfactante, caracterizada en que el anticuerpo es Cetuximab o Matuzumab.

La ClasificaciOn Internacional de Patentes (IPC) es de Ia presente solicitud es: A61 39/395.

3. De la solicitud de Patente

Reivindicaciones (artIculo 30 D 486)

El capitulo reivindicatoria de la solicitud en estudio presenta los siguientes defectos:

La reivindicaciôn I no es clara en cuanto a si la preparación contiene un solo anticuerpo o varios, puesto que menciona una "preparaciOn liofilizada de anticuerpos mono a policlonales", y la caracteriza porque el anticuerpo es Cetuximab a Matuzumab. AdemÆs,es preferible que no se incluyan nombres comerciales como lo son los referidos a dichos anticuerpos.

La amplitud de las reivindicaciones excede Ia desarrollado por el solicitante, por tanto que las propiedades fIsicas y qulmicas de los compuestos pueden variar considerablemente con la adiciôn de determinadas sustancias. Por

ejemplo, los azücares pueden comprender monosacÆridos, disacÆridoso polisacÆridos, que segün su escogencia involucran cambios profundos en la estructura de la molØcula y por consiguiente en el preparado. Por otra parte, los desarrollos y evidencias presentados, no permiten establecer los compuestos que influyen en Ia estabilidad de la preparaciOn reivindicada. Es asI coma solo se encuentran datos sobre el ejemplo 4 donde se evalUa coma azUcar, la sacarosa. Igualmente sucede con el aminoÆcido,y el surfactante que se escoja.

La reivindicaciOn 8, indica que en la preparaciOn estÆ adicionalmente "presente una concentraciôn necesaria de un agente isotônico", pero no se establece esta concentraciOn y se caracteriza el objeto por un resultado a alcanzar coma Ia es el hecho de servir "para establecer la isotonización". No existe claridad en dicha clÆusula.

Por Ia anterior, el capItulo reivindicatorio no cumple con el requisito de definiciOri, claridad, concisiOn o sustento par Ia descripciOn de acuerdo al art. 30 de la DecisiOn 486.y/o art.22 Tratado PCT.

4. Determinaciôn del Estado de la TØcnica Vkct

La fecha que se ha tenido en cuenta para determinar el Estado de la TØcnica es:

Fecha de presentación de Ia solicitud prioritaria: 21 de Diciembre de 2001.

Teniendo en cuenta la fecha indicada se realizó la bUsqueda en el estado de la tØcnica de documentos relacionados con la solicitud encontrÆndose los siguientes documentos mÆsrelevantes:

NO Documento TItulo Fecha de

publicación*
Stable lyophilized pharmaceutical
Dl W098/22136 substances from monoclonal or 28 Mayo 1998
polyclonal antibodies.
Freeze dried formulation for direct

D2 Morales et al. 99mTc-labeling ior-egf/r3 Mab: Agosto 1999 additives, biodistribution, and
stability.

5. AnÆlisis depatentabilidad (artIculo 14 D486)

Nivel inventivo (artIculol8 D486)

La reivindicaciôn I y sus dependientes reclaman una preparaciOn farmacØutica liofilizada de anticuerpos mono o policlonales que comprende 1) un azücar 0 un amino azücar, 2) un aminoÆcidoy 3) un surfactante, caracterizada en que el anticuerpo es 4) Cetuximab o Matuzumab.

El documento de estado de Ia tØcnica mÆs prôximo es DI (ver folio 146) que enseæaun mØtodo para preparar Iiofilizados que contienen 1) un azücar 0 Ufl amino azücar, 2) un aminoÆcidoy 3) un surfactante, para preparar4) anticuerpos, como agentes terapØuticos.

La ünica diferencia con DI corresponde a que la solicitud es especIfica en la escogencia de dicho anticuerpo, que en este caso es Cetuximab, el cual es un EGFR comercial, o Matuzumab. Como se evidencia durante el capItulo descriptivo, los ensayos estÆn encaminados a verificar la estabilidad de la composicion realizada mediante variación en la actividad del anticuerpo, pero no se encuentran datos que determinen un efecto sorprendente sobre el anticuerpo en cuestiOn, con otros anticuerpos, que permitan concluir que este anticuerpo en cuestión es particularmente estable con la composiciôn realizada.

Sin embargo, el documento D2 (foliosl47 153) hace referencia a preparaciones liofilizadas de anticuerpos monoclonales de que reconocen el receptor de EGF que poseen como agente estabilizador un azUcar (pagina 718, primera columna, tercer pÆrrafo), por lo que cualquier tØcnico medio en la materia hubiera podido pensar en realizar preparaciones de anticuerpos que reconocen EGFR segün las composiciones realizadas en Dl.

En este caso Dl y D2 sirven de igual manera para objetar el nivel inventivo de la solicitud en cuanto a que las caracterIsticas tØcnicas esenciales ya eran conocidas como se demostrO anteriormente.

As! pues, el problema tØcnico planteado en esta solicitud que era proveer una preparaciôn estabilizante de anticuerpos monoclonales de EGFR se deriva de las sugerencias efectuadas en las anterioridades citadas en tanto que una persona conocedora de la materia estarla motivada a realizar preparados de anticuerpos anti- EGFR como lo menciona D2 con los compuestos estabilizantes divulgados en DI. En este caso, la molØcula a estabilizar no es una caracteristica tØcnica esencial, por tanto que asI como no se demuestra un efecto sorprendente de estos anticuerpos en particular, con los estabilizantes reivindicados, tampoco se encuentra ningün argumento que Ilevara a pensar a un tØcnico que no podia emplear los adyuvantes mencionados en el documento DI con estos anticuerpos especificos.

De esta forma se establece que las reivindicaciones I a 9 no cumplen con el requisito de Nivel Inventivo.

6. Respuesta a los argumentos del solicitante

El solicitante argumenta con respecto a las objeciones realizadas sobre claridad que: "el ingrediente activo se ha limitado a Getuximab o Matuzumab;
los surfactantes se han limitado a polisorbatos y los amino azücares fueron eliminados de este pliego reivindicatorio".

A esta particular, este Despacho considera que no es cierto que los amino azücares fueran eliminados del pliego reivindicatorio como se determinó en el numeral de anÆlisis depatentabilidad, y ademÆs,en la reivindicación 1 5 no se presenta tal limitación de los surfactantes a polisorbatos coma lo establece el solicitante.

El solicitante argumenta que Ia formulaciôn de la solicitud difiere de la formulaciOn de Dl en Ia que respecta al ingrediente activo.

Este Despacho estÆ de acuerdo, enque la diferencia de Dl con la solicitud, es precisamente el ingrediente activo, y aCm cuando se evalüa la actividad de dicho ingrediente activo en el tiempo para observar su estabilidad, no se encuentra evidencia en la solicitud que determine que ünicamente el anticuerpo anti-EGFR posee dichas caracteristicas de estabilidad con la preparación de adyuvantes reivindicada. Se conoce muy bien que las tØcnicas empleadas para estabilizar compuestos activos tales como anticuerpos, involucran liofilizaciôn y el usa de azUcares, como tambiØn se evidencia en el documento D2.

Ws aCm, los datos que se presentan en el capitulo descriptivo no revelan un efecto sorprendente sobre el Estado de la TØcnica, y no se encuentra razón alguna por Ia cual el tØcnico versado que se encuentre frente al problema de estabilizar un anticuerpo anti EGFR coma el Cetuximab o Matuzumab, no utilice las enseæanzasde DI siendo dichos anticuerpos una alternativa obvia coma sujetos a ser estabilizados.

Cabe la pena resaltar que en la solicitud, no se evalüa la diferencia que existe en estabilidad de diferentes anticuerpos con las composiciones de adyuvantes formuladas, por lo que no es posible concluir si la relevancia de los receptores a los cuales se une dicha molØcula cabe en la argumentación. La problemÆtica radica en la estabilidad de una molØcula biolOgicamente activa, como un anticuerpo, no en Ia diferencia de actividades con respecto a uniOn de dichas molØculas a receptores celulares presentes en una determinada formulaciOn.

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El solicitante concluye con respecto a D2 que: "Si se tiene en cuenta que la presencia de todos estos auxiliares es esencial para poder obtener la inesperada presente formulación altamente estable, el documento D2 no
puede tenerse como una anterioridad vÆlida de la mismacapaz de afectar su
nivel inventivo".

Sin embargo, en el capItulo descriptivo, no se encuentran evidencias que determinen el nivel de afectación que poseen todos los auxiliares presentes en la formulación. Y sin embargo, este Despacho considera que el documento D2 si sugiere la estabilización del anticuerpo que reconoce EGFR mediante adiciOn de azUcar y liofihización, para que sea ütil en terapias de radio-marcaje de celulas epidØrmicas tumorales humanas. Es decir, la actividad del anticuerpo anti-EGFR continUa estable, con las formulaciones propuestas en D2; el mØtodo que se emplee para determinar su actividad no es relevante, y no se considera una evidencia que impida que D2 en combinación con Dl objete el Nivel Inventivo.

AsI mismo, este Despacho considera que no se han subsanado las objeciones presentadas en el Concepto TØcnico 1993 y no se halla en concordancia con los argumentos del solicitante.

7. Conclusion:

Los requisitos de patentabilidad de la presente solicitud se encuentran afectados asI:

No Documento NO Reivindicaciones Requisito que afecta afectadas
Dl W098/22136 1-9 Nivel Inventivo
D2 Morales et W. 1-9 Nivel Inventivo

En cumplimiento del artIculo 45 de la Decision 486 de la ComisiOn de la Comunidad Andina, se le indica que debe dar respuesta dentro del tØrmino de Go dIas contados a partir de la fecha de notificaciOn de la presente comunicaciOn. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesiôn, se denegara la patente.

NotifIquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capItulo quinto del tuIo pri7leQ de la Circular Unica NO 10 de 2001.

ALIX CARMEA CESPEDES DE VERGEL

Jefe de Divisj6n de Nuevas Creaciones

El anterior requerimiento fue

3 JUL. 2009

notificado por fijaciôn en lista, de fecha

CONCEPTO TECNICO EXAMINADOR TECNICO
No. 1990 Cadigo No. 202089