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REF: EXPEDIENTE 13-283-797

TÍTULO SOLICITADO: "FORMULACIÓN SUBCUTÁNEA DE ANTICUERPO ANTI-HER2."

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SOLICITANTE: F. HOFFMANN-LA ROCHE AG.

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TRÁMITE: REMISIÓN DE INFORMACIÓN

JOSÉ LUIS REYES VILLAMIZAR, conocido apoderado de **LAFRANCOL S.A.S.** en el asunto de la referencia, de manera respetuosa y con el propósito de coadyuvar en la decisión que sobre la solicitud divisional **13-283-797** habrá de adoptarse próximamente, me permito presentar argumentos adicionales para demostrar que el nuevo capítulo reivindicatorio presentado por el solicitante persiste en incumplir los requisitos materiales de patentabilidad y debe, por ello, ser negado al resolverse el trámite en cuestión.

I. COMPLEMENTO DE INFORMACIÓN.

En primer lugar, debe señalarse que la modificación al capítulo reivindicatorio presentada por el solicitante el 19/09/2016, en respuesta al auto notificado por fijación en lista del 28/06/2016, corresponde a un simple cambio de forma que en esa medida no logra superar los argumentos planteados en la oposición

presentada por el suscrito ni mucho menos los formulados por el examinador técnico.

1.1. Planteamiento en Función de Resultados Esperados

En primer lugar, el nuevo capítulo reivindicatorio presenta defectos de claridad, pues las reivindicaciones 1 – 7 se plantean exclusivamente en función de resultados esperados tales como ser la formulación"...***estable a la congelación y descongelación***...". Es evidente que esta redacción no define estructuralmente la invención ni permite compararla con el estado de la técnica, pues la misma se basa, como ya se ha dicho, en características esperadas resultantes de la formulación, las cuales impiden identificar los límites de la materia reclamada en las reivindicaciones 1 a 7.

1.2. Carencia de Concisión

La solicitud actualizada incumple también el requisito de concisión, por cuanto no menciona específicamente los componentes de la formulación cuando incluye una cantidad indeterminada de posibles tensioactivos no iónicos, que pueden comprender tanto polisorbatos como copolímeros de polietileno y polipropileno, circunstancia que hace indeterminada e inaceptable la redacción propuesta:

> *"4. Una formulación farmacéutica liquida, estable altamente concentrada de Trastuzumab para inyección subcutánea de acuerdo con cualquiera de las reivindicaciones 1 a 3, en donde el tensoactivo no iónico es un polisorbato seleccionado de entre el grupo que consiste de polisorbato 20, polisorbato 80 y un copolímero de polietileno y polipropileno. "*

1.3. Unidad de invención.

De igual manera, la solicitud persiste en incumplir el requisito de unidad de invención, debido a que las reivindicaciones 8 a 10 incluyen la enzima hialuronidasa, la cual es una macromolécula protéica que, en combinación con el anticuerpo trastuzumab, comprenden una actividad y efecto diferentes a los que se predican de cada una de estas sustancias por separado, lo que permite

diferenciar dos conceptos inventivos, lo que infringe lo dispuesto por el Art. 25 de la Decisión 486:

1. Una formulación farmacéutica liquida, estable, altamente concentrada de Trastuzumab para inyección subcutánea (Reivindicaciones 1 – 8), y

2. Un kit de acuerdo con la reivindicación 8 que comprende adicionalmente uno o más viales que contienen una enzima hialuronidasa (Reivindicaciones 9 y 10)

1.4. Carencia de Soporte en la Descripción

Las nuevas reivindicaciones tampoco cuentan con el debido soporte en la descripción para las modalidades que ahora se reclaman, pues no se revela allí una formulación estable de trastuzumab que no contenga la enzima hialuronidasa. Desde el inicio del trámite la formulación se ha caracterizado por contener la enzima hialuronidasa rHuPH20, como condición para la obtención de los efectos inicialmente pretendidos. Así se observa en los ejemplos 1 y 2 formulaciones A – Y, en los cuales **siempre** se empleó la referida enzima como parte esencial de la formulación.

De igual manera, no hay sustento para las reivindicaciones 8 – 10, pues en la memoria descriptiva no se observa el tratamiento que emplea un kit donde la formulación de anticuerpo se encuentra en un vial y la enzima hialuronidasa se incluya en otro, circunstancia que ratifica el flagrante incumplimiento del art. 28 de la Decisión 486.

1.5. Incumplimiento de Requisitos de patentabilidad

En adición a las objeciones planteadas, es del caso reiterar que la materia reclamada había sido previamente revelada en publicaciones disponibles en el estado de la técnica, en términos que me permito citar a continuación:

D1. US 6267958 B1 "***Protein Formulation***" *James Andya [US] Jeffrey Cleland [US], Chung C. Hsu [US] 2001-07-31*

D2. Xanthe M. Lam, Janet Yang y Jeffrey Cleland **"*Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2*"**, 1997-11-11

D3. US 2007/0071675 A1 ***"Dual variable domain immnoglobulin and uses thereof"*** *Chengbin Wu [US] Tariq Ghayur [US], Richard W. Dixon [US] 2001-07-31*

El documento D1, el cual fue citado la oposición y escritos subsecuentes, menciona todos los componentes de la formulación ahora pretendida, al referirse específicamente a formulaciones de proteínas estables liofilizadas adecuadas para administración subcutánea. En este documento, al igual que en la presente solicitud, se desarrollan formulaciones de anticuerpos anti-HER2 comunes en el estado de la técnica y rutinarios para un técnico versado en la materia respectiva.

De igual forma, D1 cita los componentes adicionales de la formulación, entre ellos sacarosa o trehalosa, histidina, metionina y el tensoactivo no iónico tal como el polisorbato, los cuales son incluidos también en el documento en trámite, sin dejar dudas de su empleo en una formulación de anticuerpo anti-HER2. Recordemos:

SUMMARY OF THE INVENTION

This invention is based on the discovery that a stable lyophilized protein formulation can be prepared using a lyoprotectant (preferably a sugar such as sucrose or trehalose), which lyophilized formulation can be reconstituted to generate a stable reconstituted formulation having a protein concentration which is significantly higher (e.g. from

(...)

acteristics of the formulation. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include; additional buffering agents; preservatives; co-solvents; antioxidants including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g. Zn-protein complexes); biodegradable polymers such as polyesters; and/or salt-forming counterions such as sodium.

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

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

Así pues, la formulación reivindicada reproduce algunos de los componentes y proporciones que aparecen en D1, e incorpora otros de uso común que una persona versada en la materia podría incoporar sin mayores dificultades:

> One useful anti-HER2 antibody pre-lyophilized formulation as discovered in the experiments detailed below was found to comprise anti-HER2 in amount from about 5–40 mg/mL (e.g 20–30 mg/mL) and sucrose or trehalose in an amount from about 10–100 mM (e.g 40–80 mM), a buffer (e.g histidine, pH 6 or succinate, pH 5) and a surfactant (e.g. a polysorbate). The lyophilized formulation was found to be

Como puede verse, se han presentado intervalos de concentración de anticuerpo anti-HER2 de 5–40mg/mL, cuando el autor del mismo documento incluye (párrafo 3, página 14) formulaciones más concentradas de dichos anticuerpos:

> 50 mg/mL. For example, the protein concentration in the reconstituted formulation maybe from about 80 mg/hn to about 300 mg/mL. Generally, the protein concentration in the reconstituted formulation is about 2–40 times greater than the protein concentration in the mixture before lyophilization.

De igual manera, D1 menciona para proteínas concentradas la siguiente formulación, la cual incluye, tanto los componentes, como cada una de las proporciones necesarias, tal como se pretende en la solicitud en trámite:

> The lyophilized protein was then reconstituted with either 4 or 20 mL of BWFI (0.9 or 1.1% benzyl alcohol) to yield concentrated protein solutions:
>
> (a) 4 mL: 102 mg/mL rhuMAb HER2, 245 mM trehalose, 21 mM sodium succinate, pH 5.0 or 21 mM histidine, pH 6.0, 0.04% Tween 20™;
>
> (b) 20 mL: 22 mg/mL rhuMAb HER2, 52 mM trehalose, 4 mM sodium succinate, pH 5.0 or 4 mM histidine, pH 6.0, 0.009% Tween 20™.

En consecuencia, la solicitud es anticipada por el desarrollo presentado en D1 bajo la selección específica de anticuerpos anti HER-2 como trastuzumab y las concentraciones específicas de formulación, que son obvias para un técnico medio versado en la materia.

De otra parte, si bien es cierto que el documento D1 no incluye la proporción exacta de metionina sí divulga expresamente su empleo, lo que permitiría a

cualquier técnico medianamente versado en la materia entrar a determinar, simplemente, los rangos requeridos de ese componente dentro de la formulación.

Por su parte, y como complemento del párrafo anterior, se observa que el documento D2 (Lam et al.,) citado por el Despacho y referido a formulaciones líquidas estables de trastuzumab que comprenden antioxidantes para la prevención de la oxidación de metionina, incluye como ejemplo 14.5mM de metionina (ver tabla 1 citada a continuación), proporción que estaría incluida dentro del intervalo de concentración divulgado por la solicitud en estudio (5–25mM de metionina). Veamos:

Table 1—List of rhuMAb HER2 Liquid Formulations

Formulation	Usage	Protein Concentration, mg/mL	Buffer Excipients
A	Single dose	5	5 mM sodium acetate, pH 5.0 147 mM NaCl 0.01% polysorbate 20
B	Multiple dose	21	5 mM sodium acetate, pH 5.0 4% mannitol 0.01% polysorbate 20 1% benzyl alcohol
C	Multiple dose	21	5 mM sodium acetate, pH 5.0 4% mannitol 0.01% polysorbate 20 1% benzyl alcohol 14.5 mM methionine
D	Multiple dose	21	5 mM sodium acetate, pH 5.0 4% mannitol 0.01% polysorbate 20 0.01% benzethonium chloride 14.5 mM methionine

Dicho documento no solo divulga sino sugiere que es necesario el empleo de metionina en la formulación por lo que su inclusión en la fórmula reivindicada era obvia en el momento de la solicitud inicial. Adicionalmente, en la solicitud en estudio no se incluye un efecto técnico aparentemente derivado de la adición de metionina en esta concentración, por lo que la selección de una concentración entre 5–25mM de metionina era obvia y derivable del estado de la técnica para obtener las reivindicaciones de la solicitud.

Un tercer documento US 2007/0071675 (Wu et al.,) citado también en la oposición, incorpora todos y cada uno de los componentes de la formulación, y en sus mismas proporciones, hecho que demuestra irrefutablemente que la solicitud

en estudio no es novedosa y sus componentes particulares no cumplen con el criterio de nivel inventivo.

Esto puede constatarse en el párrafo 184, en el cual, para una formulación parenteral, se administran de 0.1 a 250 mg/ml de proteína de unión, y en donde el buffer o tampón de histidina tienen un intervalo de concentración de 1 -50mM con un pH de 5 a 7, para luego adicionarse trehalosa u otro azúcar e incluirse metionina en un intervalo de 5–10mM y un tensioactivo como polisorbato 80 en una concentración de 0–0.05%:

[0184] The binding proteins of the invention can be incorporated into a pharmaceutical composition suitable for parenteral administration. Preferably, the antibody or antibody-portions will be prepared as an injectable solution containing 0.1-250 mg/ml binding protein. The injectable solution can be composed of either a liquid or lyophilized dosage form in a flint or amber vial, ampule or pre-filled syringe. The buffer can be L-histidine (1-50 mM), optimally 5-10 mM, at pH 5.0 to 7.0 (optimally pH 6.0). Other suitable buffers include but are not limited to, sodium succinate, sodium citrate, sodium phosphate or potassium phosphate. Sodium chloride can be used to modify the toxicity of the solution at a concentration of 0-300 mM (optimally 150 mM for a liquid dosage form). Cryoprotectants can be included for a lyophilized dosage form, principally 0-10% sucrose (optimally 0.5-1.0%). Other suitable cryoprotectants include trehalose and lactose. Bulking agents can be included for a lyophilized dosage form, principally 1-10% mannitol (optimally 2-4%). Stabilizers can be used in both liquid and lyophilized dosage forms. principally 1-50 mM L-Methionine (optimally 5-10 mM). Other suitable bulking agents include glycine, arginine, can be included as 0-0.05% polysorbate-80 (optimally 0.005-0.01%). Additional surfactants include but are not limited to polysorbate 20 and BRIJ surfactants. The pharmaceutical composition comprising the binding proteins of the invention prepared as an injectable solution for parenteral administration, can further comprise

Este documento incluye anticuerpos anti-HER2 como el trastuzumab como anticuerpos parentales de estas formulaciones.

[0112] Parent monoclonal antibodies may also be selected from various therapeutic antibodies approved for use, in clinical trials, or in development for clinical use. Such therapeutic antibodies include, but are not limited to, rituximab (Rituxan®, IDEC/Genentech/Roche) (see for example U.S. Pat. No. 5,736,137), a chimeric anti-CD20 antibody approved to treat Non-Hodgkin's lymphoma; HuMax-CD20, an anti-CD20 currently being developed by Genmab, an anti-CD20 antibody described in U.S. Pat. No. 5,500,362, AME-133 (Applied Molecular Evolution), hA20 (Immunomedics, Inc.), HumaLYM (Intracel), and PRO70769 (PCT/US2003/040426, entitled "Immunoglobulin Variants and Uses Thereof"); trastuzumab (Herceptin®, Genentech) (see for example U.S. Pat. No. 5,677,171), a humanized anti-Her2/neu antibody approved to treat breast cancer; pertuzumab (rhuMab-2C4, Omnitarg®), currently being developed by Genentech; an anti-Her2 antibody described in U.S. Pat. No. 4,753,894; cetuximab (Erbitux®, Imclone) (U.S. Pat. No. 4,943,533; PCT WO 96/40210), a chimeric anti-

Así las cosas, se reitera que la modificación realizada no supera las objeciones de novedad y nivel inventivo ya establecidas, en términos que serán resumidos a continuación:

Presente solicitud divisional –Nuevo capítulo reivindicatorio	**D3:** US 2007/0071675	**D1: US 6.267.958**
1. Una formulación farmacéutica liquida, estable, altamente concentrada de Trastuzumab para inyección subcutánea que consiste esencialmente de:	*Una formulación farmacéutica líquida, estable, altamente concentrada de un anticuerpo farmacéuticamente activo anti-HER2 para inyección subcutánea que consiste de:*	*Una formulación farmacéutica líquida, estable, altamente concentrada de un anticuerpo farmacéuticamente activo anti-HER2 para inyección subcutánea que consiste de:*
entre 120 ± 18 mg/ml de Trastuzumab;	*0.1- 250 mg/ml (dentro del intervalo 120±18 mg/ml) de anti-HER2*	*102 mg/ml (dentro del intervalo 120±18 mg/ml) de rhuMAb HER2*
entre 1 a 50 mM de un tampón de histidina que proporciona un pH de 5,5 ± 0,6;	*1 a 50 mM de histidina pH 5 - 7 (dentro del intervalo pH 5,5 ±0,6)*	*21 mM de histidina pH 6.0 (dentro del intervalo pH 5,5 ±0,6)*
entre 15 a 250 mM de α,α-trehalosa dihidratada o	*trehalosa*	*245 mM de trehalosa*

sacarosa y		
entre 5 a 25 mM de metionina; y	*5 – 10 mM de Metionina*	*Metionina*
entre 0,01 a 0,08% de un tensioactivo no iónico;	*0- 0,05% de tensioactivo no iónico (Polisorbato-80)*	*0,04% de Tween 20 (tensoactivo no iónico)*

De acuerdo con las diferencias en selección de proporciones de los componentes tal como metionina, los documentos D1 y D2 permitían derivar la materia de la solicitud bajo los mismos componentes y proporciones ahora pretendidos.

Es importante mencionar que la formulación bajo criterios de diferentes concentraciones de ingrediente activo y otros parámetros a definir como estabilización o actividad en el tiempo, permiten al técnico medio realizar ajustes con el fin de encontrar variaciones en las proporciones, sin que esto implique un salto cuantitativo sobre las formulaciones ya existentes, pues ello simplemente es lo que permite desarrollar protocolos de estandarización bajo condiciones diferentes.

1.6. Referencia a los argumentos del solicitante.

En su respuesta, el solicitante menciona que *la formulación no es igual a lo divulgado por el documento D1 en que la formulación farmacéutica comprende puntualmente el anticuerpo anti-HER2 «Trastuzumab» en una cantidad de «120 ± 18 mg/ml». Además, dicho documento no revela la específica combinación de excipientes y concentraciones de la formulación reclamada en la solicitud en estudio. Por consiguiente, me permito manifestar que la presente solicitud cumple a cabalidad con los requisitos de patentabilidad establecidos en el Artículo 16 de la Decisión Andina 486/2000.*

Frente a este argumento es claro que el solicitante pasa por alto que una generalización como anticuerpo anti-HER2 INCLUYE el anticuerpo trastuzumab y que además siendo un anticuerpo comercialmente conocido, la relevancia es mayor como se observa además en el documento citado D3, que define el anticuerpo anti-HER2 como trastuzumab específicamente.

Acierta el Despacho, por cuanto el anticuerpo trastuzumab solo corresponde a una selección de la fórmula general anticuerpo anti-HER2 por lo que ambas denominaciones en este caso son iguales. En adición a ello, la concentración de proteína en D1 en la formulación puede variar de 80 mg/mL a 300 mg/mL, concentración incluida e indicada en la reivindicación 1.

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

De otra parte, en cuanto a la divulgación de los demás excipientes y proporciones, en la tabla de comparación antes señalada, se observa que únicamente la proporción de metionina no se ha divulgado; sin embargo y tal como se mencionó, se observa de D3 que esta concentración es común y rutinaria en la técnica con lo que no hay lugar a dudas de que la solicitud no es novedosa frente a D1 o D3, cuya combinación, además, resulta obvia para una persona versada en la materia.

Por otro lado, el solicitante afirma que la diferencia entre el documento D1 y la invención radica en tres aspectos:

> *(i). El documento D1 no describe una formulación líquida de anticuerpo anti-HER2 altamente concentrado.*
> *(ii). El documento D1 no divulga o sugiere las cantidades exactas de Trastuzumab y excipientes en la formulación líquida como las reclamadas en la presente solicitud, y en particular, no hace referencia a las cantidades de metionina útiles en una formulación de este tipo.* □
> *(iii). El documento D1 no describe ningún dispositivo de inyección o kit que comprenda la formulación líquida de la presente invención y una enzima hialuronidasa en una formulación separada.* □

Sin embargo, como ya se ha mencionado, pese a que D1 menciona efectivamente composiciones liofilizadas, también revela de qué manera se manifiesta la

formulación posterior luego de reconstituirse, es decir, como una formulación líquida de anticuerpo altamente concentrado y que puede contener de 80 a 300 mg/ml.

En lo que hace a los excipientes y cantidades exactas en la solicitud y cantidades de metionina útiles, ya se ha mencionado que la solicitud emplea que están incluidos entre los que enseña el arte anterior, siendo incluso, prácticamente los mismos revelados en D3. En este caso, la única diferencia que se observa es la cantidad exacta de metionina, que útilmente se ha incluido en la reivindicación 1, siendo estas cantidades normales en el estado de la técnica como se observa también en D2 y D3.

Ahora bien, aunque D1 no mencione los kits que comprende formulaciones líquidas y una enzima hialuronidasa en una formulación separada, la solicitud en sus ejemplos tampoco lo hace, pues todo el desarrollo de la solicitud se basa en la combinación del anticuerpo anti-HER2 y la enzima hialuronidasa rHuPH20. En este orden de ideas, se reitera que la solicitud viola flagrantemente el Articulo 25 sobre unidad de invención y el Articulo 30 al no cumplir con el respectivo soporte en la descripción para formulaciones que no comprenden la enzima hialuronidasa tal como se expresó a la largo del trámite.

En conclusión D1 y D3 en combinación con D2 demuestran que no existe nivel inventivo en la solicitud en trámite para las formulaciones de trastuzumab que incluyan metionina en las proporciones reivindicadas, por cuanto no existen efectos sorprendentes en las mismas, ni evidencia de ventajas técnicas comprobadas sobre las formulaciones de anticuerpos del estado de la técnica.

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II. PRUEBAS.

Ruego que se tengan como pruebas de este escrito, los documentos mencionados en el texto del mismo, todos ellos aportados por el suscrito y obrante en el expediente, y el siguiente:

- Lam, X. M., Yang, J. Y. and Cleland, J. L. (1997), "**Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2"**. J. Pharm. Sci., Vol. 86, No. 11. doi:10.1021/js970143s, con fecha de publicación 03 de agosto de 1997. Páginas 1250–1255.

Señor Superintendente,

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Antioxidants for Prevention of Methionine Oxidation in Recombinant Monoclonal Antibody HER2

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Received April 10, 1997, from the *Department of Pharmaceutical Research and Development, Genentech, Inc., 460 Pt. San Bruno Blvd., MS 10, South San Francisco, CA 94080.* Accepted for publication August 3, 1997[⊗].

Abstract □ Recombinant humanized monoclonal antibody HER2, rhuMAb HER2, in liquid formulations undergoes oxidation when exposed to intense light and elevated temperatures (30 & 40 °C). Met-255 in the heavy chain of the Fc region of the antibody is the primary site of oxidation. Met-431 of the Fc fragment can also be oxidized under extreme conditions. The amount of oxidation was determined by cleaving the Fab and Fc fragments by papain digestion, and the oxidized Fc fragment was detected by hydrophobic interaction chromatography. Oxidation of rhuMAb HER2 was also formulation dependent. The presence of NaCl in the rhuMAb HER2 formulation caused an increase in oxidation at higher temperatures after contact with stainless steel containers or stainless steel components in the filling process. The corrosion of stainless steel by chloride ions at the low pH of the formulation buffer generated iron ions that catalyzed methionine oxidation in rhuMAb HER2. Temperature-induced oxidation of rhuMAb HER2 occurred by the formation of free radicals, and light-induced oxidation of rhuMAb HER2 occurred via singlet oxygen pathway. Antioxidants, such as methionine, sodium thiosulfate, catalase, or platinum, prevented Met oxidation in rhuMAb HER2, presumably as free radicals or oxygen scavengers. The minimum effective levels (molar ratios of protein to antioxidant) required to inhibit temperature-induced oxidation were 1:5 and 1:25 for methionine and thiosulfate, respectively. A thiosulfate adduct of rhuMAb HER2 was observed by cation-exchange chromatography. These studies demonstrate that stoichiometric amounts of methionine and thiosulfate are sufficient to eliminate temperature-induced oxidation of rhuMAb HER2 caused by free radicals that were generated by the presence of metal ions and peroxide impurities in the formulation.

Introduction

Oxidation of methionine is one of the major degradation pathways in many protein pharmaceuticals, including interleukin-2, relaxin, parathyroid hormone, and human growth hormone.[1–7] Methionine residues in proteins are susceptible to oxidation, resulting in the formation of methionine sulfoxide and, under extreme conditions, sulfones. Methionine sulfoxide formation can occur during synthesis, purification, formulation, manufacturing, and storage of protein pharmaceuticals. During formulation and storage, oxidation of methionine-containing proteins can be caused by the presence of certain formulation excipients, including polyethylene glycols and nonionic polyether surfactants; these compounds can undergo autooxidation to form peroxides.[8] In fact, peroxides such as hydrogen peroxide have been widely used for studying the kinetics and mechanisms of methionine oxidation in proteins.[9–11] Peroxides react with metal ions to form free radicals that can initiate oxidation of proteins.[12,13] Methionine can also be photooxidized by a free radical pathway,[14] or via singlet oxygen intermediate formation.[15] For instance, photosensitizers can absorb radiation energy to form excited species that initiate the formation of free radicals for methionine oxidation. The excited species can also react with oxygen to form singlet oxygen, which in turn oxidizes methionine to yield methionine sulfoxide. Photolytic degradation of protein drug products can be influenced by many factors, including the buffer and its concentration, excipients, and formulation pH. Storage conditions, such as radiation intensity and duration and temperature, can also affect the rate of photolytic degradation.

The use of antioxidants in pharmaceutical products is one of the common ways to protect the drugs from oxidative degradation.[16,17] The unknown toxicity of many antioxidants and their incompatibility with proteins and/or excipients in parenteral formulations has hampered their use in protein pharmaceuticals. Nevertheless, several antioxidants have been identified for the prevention of methionine oxidation in recombinant human proteins,[18] including chelating agents, reducing agents, oxygen scavengers, and chain terminators.[19] Chelating agents bind to metal ions that catalyze oxidative reactions. Metal ions can react with peroxide impurities or the protein itself in the formulation to form free radicals that initiate oxidative degradation. Reducing agents reduce an oxidized drug product. For instance, the oxidized form of methionine, methionine sulfoxide, can be reduced to methionine by a reducing agent. Oxygen scavengers are substances that are more susceptible to oxidation than the drugs they are protecting. These substances are reducing agents that can react with oxygen by preferential oxidation, and thus remove the source of oxidation. Chain terminators are substances that can react with free radicals to produce intermediates that terminate the oxidation reactions via free radical pathways.

The overexpression of a 185 kDa glycoprotein ($p185^{HER2}$) has been found in tumor cells of breast and ovarian cancer, and rhuMAb HER2 binds to the extracellular domain of the glycoprotein to inhibit the growth of human breast carcinoma cells.[20,21] In the initial development of a liquid formulation for rhuMAb HER2 used in the treatment of breast cancer patients, methionine sulfoxide formation was detected using *tert*-butylhydroperoxide oxidant.[22] The goals of this study were to investigate the effect of temperature and light on oxidation of rhuMAb HER2 in liquid formulations, to determine the mechanisms of methionine oxidation, and to identify antioxidants for their prevention.

Experimental Section

Materials— rhuMAb HER2, expressed in Chinese hamster ovary cells, was purified by Process Recovery Operations at Genentech, Inc. All chemicals and reagents used were reagent grade. Solvents used for hydrophobic interaction and cation-exchange chromatography (HPLC) assays were filtered before use. *L*-Methionine, sodium thiosulfate, and catalase were purchased from Sigma Chemical Company (St. Louis, MO). Platinum was purchased from Aldrich Chemical Company (Milwaukee, WI). Carboxypetidase B and papain (from Carcia papaya) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

rhuMAb HER2 Formulations—The thermal stability and photostability studies of rhuMAb HER2 were assessed for the single and multiple dose formulations listed in Table 1. Formulation A was designed for use in single dosing, and Formulations B–D were

[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1997.

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Table 1—List of rhuMAb HER2 Liquid Formulations

Formulation	Usage	Protein Concentration, mg/mL	Buffer Excipients
A	Single dose	5	5 mM sodium acetate, pH 5.0 147 mM NaCl 0.01% polysorbate 20
B	Multiple dose	21	5 mM sodium acetate, pH 5.0 4% mannitol 0.01% polysorbate 20 1% benzyl alcohol
C	Multiple dose	21	5 mM sodium acetate, pH 5.0 4% mannitol 0.01% polysorbate 20 1% benzyl alcohol 14.5 mM methionine
D	Multiple dose	21	5 mM sodium acetate, pH 5.0 4% mannitol 0.01% polysorbate 20 0.01% benzethonium chloride 14.5 mM methionine

designed for multiple does and therefore contain preservatives. All formulations were filled under aseptic conditions into 10-mL glass vials with a fill volume of 10 mL.

HPLC Assays—A Hewelett Packard HP-1090L HPLC instrument equipped with a diode array detector and solvent delivery system was used to assess the stability of rhuMAb HER2 by hydrophobic interaction, size exclusion, and cation-exchange chromatography methods.

Hydrophobic interaction HPLC (HIC) was employed to quantify the amount of oxidized Fc in rhuMAb HER2. For separating the Fc domain from the Fab fragment of the monoclonal antibody, rhuMAb HER2 samples were first digested with carboxypeptidase B at a concentration of 1:100 (w/w; carboxypeptidase B: rhuMAb HER2) at 37 °C for 20 min, followed by papain digestion at a concentration of 1:200 (w/w; papain: rhuMAb HER2) at 37 °C for 2 h. Digested samples were injected directly onto a TSK Butyl-NPR column (4.6 × 35 mm, Tosohaas) and eluted at 0.5 mL/min with a mobile phase consisting of buffer A (20 mM Tris at pH 7.0) and buffer B (2 M ammonium sulfate in buffer A). A linear gradient from 10 to 100% buffer A was performed over 37 min at ambient temperature. Peak detection was performed at 214 nm.

Native size exclusion chromatography (SEC) was used to determine the amount of soluble aggregates and monomer present in the rhuMAb HER2 formulations. Samples were eluted isocratically at ambient temperature using a TSK G3000SWXL column (7.8 × 300 mm, Tosohaas) at 1 mL/min with phosphate buffered saline (PBS) at pH 7.2 as mobile phase. The runtime was 20 min, and peaks were detected at 280 nm.

Ion exchange HPLC (IEC) was employed with a Bakerbond WP Carboxy Sulfon column (4.6 × 250 mm, J. T. Baker) to characterize deamidation of rhuMAb HER2 in the formulations. Samples were eluted at 1 mL/min with mobile phase consisting of buffer A (20 mM sodium phosphate at pH 6.9) and buffer B (0.2 M sodium chloride in buffer A). A linear gradient from 10 to 45% buffer B was performed over 55 min. The column was maintained at 40 °C, and peaks were detected at 214 nm.

Extracellular Domain (ECD) Plate Binding Assay—This assay assessed the ability of rhuMAb HER2 to bind to the ECD of the $p185^{HER2}$ glycoprotein. rhuMAb HER2 samples were diluted into assay buffer to concentrations within the linear range (10–70 ng/mL) of the standard curve for the assay. Diluted samples were incubated with recombinant $p185^{HER2}$ ECD protein in 96 wells of a microtiter plate for 1 h at ambient temperature. After incubation, the wells were washed with assay buffer, and a HRP-conjugated anti-human Fc goat antibody was added. After additional washing, a substrate of *o*-phenylenediamine was added for a 10 min incubation at ambient temperature in the dark. Absorbances at 490–492 nm were read, and sample concentrations were determined from a standard curve using a four-parameter logistic curve fitting program.

Bioassay—This assay quantitated the bioactivity of rhuMAb HER2 by measuring the antiproliferative effects of the antibody on BT-474 cells derived from human breast ductal carcinoma. rhuMAb HER2 samples were diluted into assay buffer to a concentration of 0.25 μg/mL. Diluted samples (100 μL) were incubated with BT-474 cells in a 96-well tissue culture plate at 37 °C for 96 h. After incubation, medium was removed and the plate was stained with Crystal Violet stain. Bioactivity of rhuMAb HER2 was quantitated by measuring the absorbance at 540 nm. Sample concentrations were determined from a four-parameter fit equation generated from the standard curve data. The range for sample quantitation was 20% from the low and high asymptote of the curve generated.

Thermal Stability Studies—The effect of temperature on oxidation of rhuMAb HER2 was studied by incubating samples of Formulation A (single dose) and Formulation B (multiple dose) at 5, 30, and 40 °C for 2 weeks. At each timepoint, samples were analyzed for oxidation by HIC, aggregation by SEC, deamidation by IEC, and activity by ECD plate binding assay and bioassay.

Photostability Studies—Two vials of each liquid formulation (A–D) were stored in a light box (Forma Scientific, model 3890) under high intensity fluorescent light maintained at 20 000 lux, which is ~15–20 times that of indoor fluorescent light. Another two vials wrapped with aluminum foil were also stored in the light box as controls for the same period of time. The temperature of the light box was at 27 °C. After 2 weeks, samples were assayed by the same analytical methods as those used for the thermal stability studies.

Antioxidant Studies—To study the inhibitory effect of antioxidants on light-induced oxidation of rhuMAb HER2, methionine and sodium thiosulfate were added to formulation A at final concentrations of 3.5 mM (0.05% w/v) and 6.3 mM (0.1% w/v), respectively. The antioxidant-containing formulations were filtered into 10-mL glass vials with a fill volume of 10 mL, and two vials from each formulation were kept in the light box. As controls, two other vials were shielded from light by wrapping with aluminum foil and stored in the same light box. At 2 weeks, samples were analyzed for oxidation by the HIC assay.

The inhibitory effect of antioxidants on temperature-induced oxidation of rhuMAb HER2 was evaluated by adding 3.5 mM methionine (0.05%), 6.3 mM sodium thiosulfate (0.1%), catalase (0.002%), and platinum (0.005%) to Formulation A before filling into 10-mL glass vials. Samples were stored at 40 °C for 2 weeks and then assayed by the HIC method. The effect of molecular oxygen on oxidation of rhuMAb HER2 was also examined by replacing headspace oxygen from two 10-mL filled vials of Formulation A with nitrogen before they were stored at 40 °C for 2 weeks. Samples were analyzed by the HIC assay.

To determine the minimum effective levels required to inhibit rhuMAb HER2 oxidation, methionine and sodium thiosulfate at molar ratios between 1:1 and 1:180 (protein: antioxidant) were added to Formulation A. Antioxidant-containing rhuMAb HER2 material was then filled into 10-mL glass vials. Samples were stored at 40 °C for 2 weeks, and then assayed for oxidation by the HIC method.

Results and Discussion

Thermal Stability of rhuMAb HER2—The effect of temperature on oxidation of rhuMAb HER2 was studied in Formulation A (single dose) and Formulation B (multiple dose) at 5, 30, and 40 °C. As shown in Figure 1, the increase in oxidation of rhuMAb HER2 was temperature and formulation dependent. After incubation for 2 weeks, the rhuMAb HER2 in Formulation A (5 mg/mL protein in 5 mM sodium acetate, 147 mM NaCl, 0.01% polysorbate 20, pH 5.0) had 10, 17, and 52% oxidized Fc at 5, 30, and 40 °C, respectively. The percentage of oxidized Fc analyzed by HIC at each timepoint was defined as the sum of the peak areas of the two oxidized peaks (1 & 2) divided by the total peak areas of the Fc peaks (Figure 2). Previous studies demonstrated that peak 1 contained oxidized Met-255 and peak 2 consisted of rhuMAb HER2 oxidized at both Met-255 and Met-431 in the Fc.[22] Although these two methionine residues located on the surface

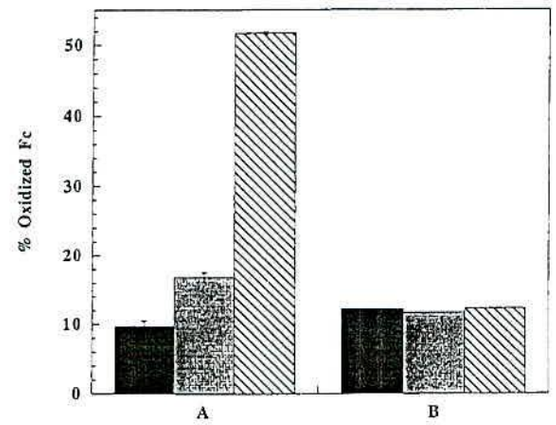


Figure 1—Effect of temperature on oxidation of rhuMAb HER2 in Formulations A and B. Samples were analyzed for methionine oxidation by HIC after 2 weeks of incubation at 5 °C (black), 30 °C (gray), and 40 °C (striped).

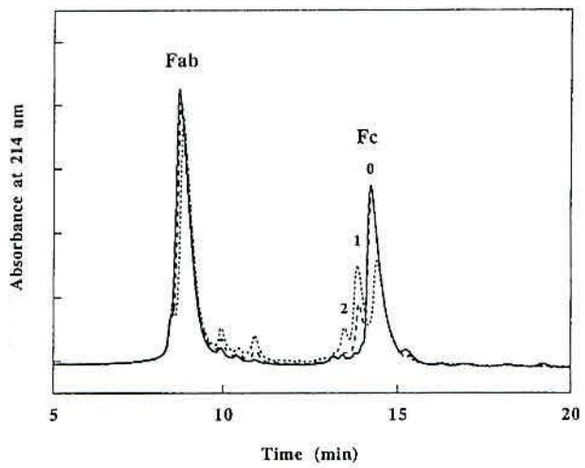


Figure 2—HIC chromatograms showing the effect of temperature on oxidation of the two methionine residues on the Fc domain of rhuMAb HER2 (Formulation A). Samples were incubated at 5 °C (solid line), 30 °C (dashed line), and 40 °C (dotted line) for 2 weeks. The three main peaks of the rhuMAb HER2 Fc domain are non-oxidized Fc (peak 0), Fc fragment with oxidized Met-255 (peak 1), and Fc fragment with oxidized Met-255 and Met-431 (peak 2). After incubation, the 5, 30, and 40 °C samples contained 10, 17, and 52% oxidized Fc (peaks 1 & 2), respectively.

of the antibody are more susceptible to oxidation than other methionine sites that are buried in the variable domains, they are neither within the Fcγ receptor region nor the complement C1q epitopes in the Fc of the heavy chain. In Formulation A, oxidation of these two methionine residues increased with temperature. In contrast, Formulation B (multiple dose), which consisted of different excipients, did not exhibit an increase in methionine oxidation at high temperatures (30 & 40 °C) after 2 weeks of incubation. In addition, when the surfactant (polysorbate 20) was not added in Formulaiton A, <10% oxidized Fc was detected in the samples that were strored at 30–40 °C. These results indicate that oxidation of rhuMAb HER2 was also formulation dependent.

The difference in the degree of oxidation of rhuMAb HER2 in Formulations A and B at high temperatures was also process related. The NaCl-containing rhuMAb HER2 Formulation A had 52% oxidized Fc at 40 °C for 2 weeks when it was filled into 10-mL glass vials by a stainless steel filler, as compared with 18% oxidized Fc when filled by a nonstainless steel filler. The replacement of NaCl by mannitol as a tonicifier yielded Formulation B that did not induce rhuMAb HER2 oxidation at high temperatures, even though it was filled by the same stainless steel filler. These results reveal that the excipient, NaCl, in Formulation A played an important role in rhuMAb HER2 oxidation. One hypothesis for the role of salt in the oxidation of rhuMAb HER2 was the corrosion of the stainless steel components of the filler by chloride ions at low pH, generating metal ions such as iron that catalyzed oxidation. When rhuMAb HER2 in Formulation A was tested for metal ions by induced coupled plasma (ICP) spectrophotometry, the amount of iron increased from <0.1 ppm in the control sample (glass vial) to 3.1 ppm in a sample stored at 5 °C for 3 months in a 30-mL stainless steel container (Type 316L, Fluid Line Technology). Furthermore, when the NaCl-containing rhuMAb HER2 Formulation A was prepared by diluting its concentrated bulk with a formulation buffer that had been made in a stainless steel tank, the protein had a 26% increase in oxidation after 2 weeks at 40 °C. No increase in oxidation was observed for the formulation buffer prepared in glass container. These results further reveal that the presence of NaCl in a formulation and contact with stainless steel both contributed to the increase in rhuMAb HER2 oxidation, probably through the generation of metal ions.

To further confirm that the formation of iron ions as a result of corrosion of stainless steel by the salt was the major cause for the oxidation of rhuMAb HER2 at high temperatures, a metal chelating agent (EDTA) was added to the sample vials filled by stainless steel filler prior to incubation. After incubation at 40 °C for 2 weeks, samples containing either 0.02 or 0.05 mM EDTA did not show an increase in methionine oxidation, as compared with a 42% increase in the control sample without the chelating agent. This result suggests that the formation of iron ions in the rhuMAb HER2 Formulation A due to the contact with stainless steel was responsible for the oxidation of the protein at high temperatures.

The oxidation of methionine residues in peptides and proteins is often initiated through the generation of reactive oxygen species,[23] and the rate and amount of methionine sulfoxide formation are affected by exogenous factors such as metal ions.[24] Although many oxidative pathways are possible for methionine, the mechanism for the temperature-induced oxidation of methionine residues of rhuMAb HER2 in the NaCl-containing Formulation A may involve metal-ion-catalyzed free radical formation. The formulation contained polysorbate 20, a nonionic polyether surfactant that can undergo autooxidation upon storage to form alkyl hydroperoxides. These peroxide impurities can be further decomposed by the presence of heavy metal ions, such as iron formed by the contact between stainless steel, and chloride ion at low pH to initiate alkoxyl free radical (RO•). This alkoxyl free radical can then react with methionine residues of rhuMAb HER2 to form a positively charged methionine free radical that can further react with the hydroxide ion to generate hydroxyl methionine free radical in the propagation phase. The alkoxyl free radical can also react with molecular oxygen to generate hydroxyl radical ($HO_2^{\bullet}$), which can be terminated by reacting with the hydroxyl methionine free radical to form methionine sulfoxide, as shown in the following proposed mechanism:

Initiation

$$ROOH + Fe^{2+} \rightarrow RO^{\bullet} + Fe^{3+} + OH^{-} \quad (1)$$

Propagation

$$RO^{\bullet} + R'\text{-}S\text{-}CH_3 \rightarrow R'\text{-}S^{\bullet}_{+}\text{-}CH_3 + RO^{-}$$

$$R'\text{-}S^{\bullet}_{+}\text{-}CH_3 + OH^{-} \rightarrow R\text{-}S^{\bullet}(OH)\text{-}CH_3 \quad (2)$$

$$RO^{\bullet} + O_2 + H_2O \rightarrow HO_2^{\bullet} + ROOH$$

Termination

$$R\text{-}S^{\bullet}(OH)\text{-}CH_3 + HO_2^{\bullet} \rightarrow R\text{-}S(O)\text{-}CH_3 + H_2O_2 \quad (3)$$

Table 2—Effect of Temperature on Stability of rhuMAb HER2 Liquid Formulations

Temp (°C)	Days	Formulation	% Monomer (SEC)	% Deamidation (IEC)	% Specific Activity (ECD Assay)	% Specific Activity (Bioasay)
5	0	A	100	9.4	104.6 ± 2.4	94.9 ± 10.6
		B	100	12.0	94.8 ± 2.1	113.0 ± 18.1
30	14	A	99.4	8.6	98.0 ± 3.1	93.5 ± 6.1
		B	99.4	8.3	107.7 ± 3.2	104.0 ± 4.5
40	14	A	99.0	8.4	91.9 ± 3.9	96.8 ± 3.0
		B	99.6	6.7	98.5 ± 0.1	97.5 ± 8.3

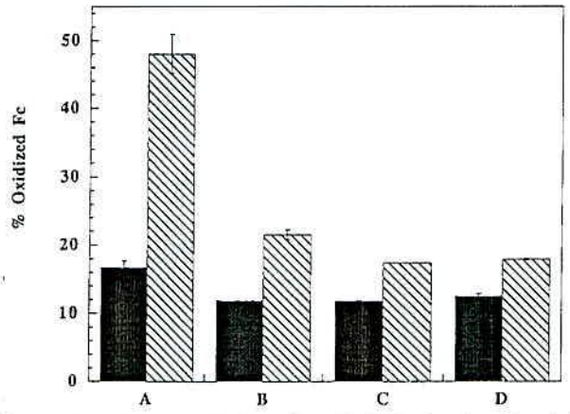


Figure 3—Effect of light on oxidation of rhuMAb HER2 in various formulations. Wrapped (black) and unwrapped (striped) samples were stored in a ligth box with light intensity of 20 000 lux for 2 weeks. The light box temperature was 27 °C. After light exposure, samples were analyzed for methionine oxidation by HIC.

The polysorbate 20 was necessary to prevent agitation-induced aggregation of the protein, so it could not be excluded so as to eliminate oxidation by the pathways described in eqs 1–3. Although temperature and excipient in the formulation could cause methionine oxidation of rhuMAb HER2, they had no significant effect on aggregation, deamidation, and biological activity of the protein (Table 2). In fact, rhuMAb HER2 in Formulation A, which contained 52% oxidized Fc, still retained >90% of its binding activity and bioactivity, as determined by the plate binding assay and bioassay, respectively. This result is probably due to the fact that the potential oxidation sites are neither within the complementarity-determining regions (CDRs) nor the Fc receptor binding sites of the antibody. The major concern about methionine oxidation in rhuMAb HER2 is related to regulatory issue. To fulfill the FDA requirement for a stable formulation, one must demonstrate that the pharmaceutical product is not >10% degraded and that the degradation products do not have any adverse effects on the safety and efficacy of the drug.

Photostability Studies—To study an alternative oxidation pathway for rhuMAb HER2, the effect of light on methionine oxidation in rhuMAb HER2 was assessed in various formulations. The results are shown in Figure 3. After 2 weeks of storage in the light box, the unwrapped vials of rhuMAb HER2 in formulations B, C, and D all had a slight increase in oxidized Fc (17–22%) as compared with ~12% in their wrapped control vials. However, the amount of oxidation induced by light in these formulations was still much less than that observed in Formulation A (48% oxidized Fc in the unwrapped vials and 16.5% in the wrapped control vials). These results suggest that rhuMAb HER2 was susceptible to photooxidation in all formulations tested, and that the greater extent of methionine oxidation in Formulation A was probably due to the combined effects of light and the increase in temperature of the light box (27 °C).

Photolytic oxidation of rhuMAb HER2 can occur when the protein itself absorbs energy from the radiation source (fluorescence light in the light box). This absorbed energy can be dissipated in the form of thermal energy, which produces an increase in temperature for inducing methionine oxidation of rhuMAb HER2. This sequence may also be an explanation for the greater increase in methionine oxidation in Formulation A than in Formulations B, C, and D after light exposure, because Formulation A was shown to be temperature sensitive (Figure 1). Another mechanism for photolytic degradation of rhuMAb HER2 in these formulations may involve the formation of singlet oxygen, which has shown to be one of the photooxidation pathways for methionine-containing compounds.[15] The formation of singlet oxygen was probably due to the presence of polysorbate 20, a photosensitive agent, in the rhuMAb HER2 formulations. After the absorption of radiation energy from the light box, polysorbate may dissipate its energy by reacting with molecular oxygen to generate singlet oxygen. This singlet oxygen can react with methionine to form an intermediate that oxidizes a second methionine molecule to form sulfoxide, as shown in the following equations:

$$\text{Polysorbate} \xrightarrow{h\nu} \text{Polysorbate}^* \quad (4)$$

$$\text{Polysorbate}^* + O_2 \rightarrow \text{Polysorbate} + {}^1O_2 \quad (5)$$

$$\text{R-S-CH}_3 + {}^1O_2 \rightarrow \text{R-S(OO)-CH}_3 \quad (6)$$

$$\text{R-S(OO)-CH}_3 + \text{R-S-CH}_3 \rightarrow \text{R-S(O)-CH}_3 + \text{R-S(O)-CH}_3 \quad (7)$$

The photo-induced oxidation of rhuMAb HER2 was prevented by the presence of methionine in the formulation. As shown in Figure 3, Formulation B (21 mg/mL rhuMAb HER2 in 5 mM sodium acetate, pH 5.0, 4% mannitol, 0.01% polysorbate 20, and 1% benzyl alcohol) contained 22% oxidized Fc after 2 weeks of light exposure as compared with 17% in Formulation C, which consisted of the excipients used in Formulation B plus 14.5 mM methionine. Formulation D, which contained 0.01% benzethonium chloride as preservative, photo-oxidized at a similar rate as Formulation C, which contained 1% benzyl alcohol. Thus, the preservative did not affect the rate or extent of oxidation. These results suggest that the presence of a reducing agent such as methionine can act as an antioxidant to reduce photooxidation of methionine in the protein. In fact, methionine has been shown to be effective in reducing methionine sulfoxide formation in peroxide-mediated oxidation of recombinant human ciliary neurotrophic factor and nerve growth factor.[18]

In summary, methionine oxidation was the major degradation pathway of rhuMAb HER2 caused by light. The oxidized rhuMAb HER2 (light-induced) appeared to retain its full binding activity and bioactivity in the plate binding assay and bioassay, respectively. Light had no significant effect on

Table 3—Effect of Light on Stability of rhuMAb HER2 Liquid Formulations

Condition	Days	Formulation	% Monomer (SEC)	% Deamidation (IEC)	% Specific Activity (ECD Assay)	% Specific Activity (Bioasay)
Dark	14	A	99.4	8.3	118.0 ± 6.8	NT[a]
		B	100	9.4	93.1 ± 9.5	104.7 ± 4.5
		C	100	9.8	88.7 ± 5.4	106.4 ± 4.7
		D	100	9.6	90.1 ± 0.9	111.7 ± 5.8
Light	14	A	98.6	8.0	113.7 ± 5.2	NT
		B	100	8.2	95.0 ± 7.6	93.2± 6.3
		C	100	8.2	100.6 ± 1.4	98.8 ± 4.6
		D	100	8.2	98.6 ± 6.9	95.0 ± 0.7

[a] NT = not tested.

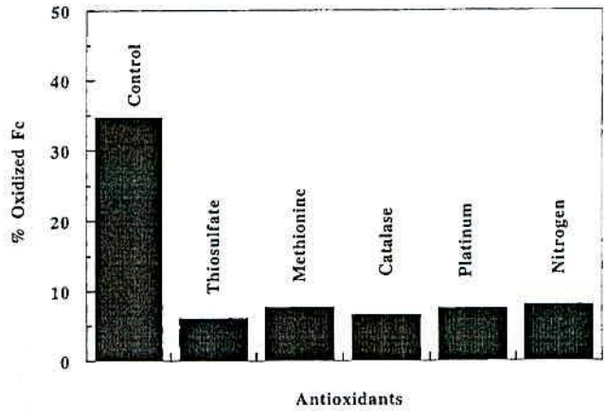


Figure 4—Methods to prevent temperature-induced oxidation of rhuMAb HER2 in Formulation A. Antioxidants were added to the formulation before filling into sample vials for incubation at 40 °C for 2 weeks. After incubation, samples were assayed by HIC to assess methionine oxidation. The amount of oxidation was compared with a control sample containing no antioxidant stored under the same conditions.

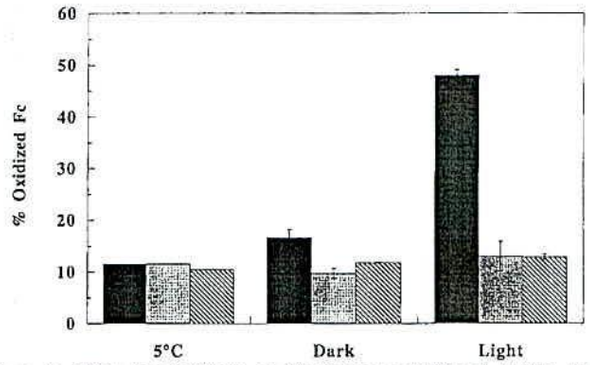
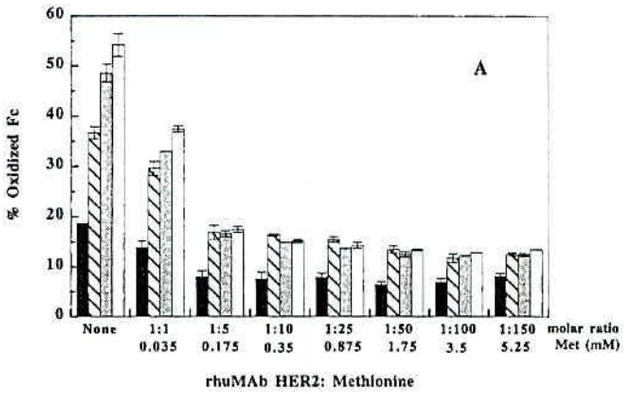


Figure 5—Effect of antioxidants on light-induced oxidation of rhuMAb HER2 Formulation A. Antioxidants were added to the formulation before filling into sample vials. Sample containing no antioxidant (black), 6.3 mM sodium thiosulfate (gray), and 3.5 mM methionine (striped) were stored wrapped (Dark) and unwrapped (Light) in a light box with light intensity of 20 000 lux for 2 weeks. The light box temperature was 27 °C. After light exposure, samples were assessed for methionine oxidation of rhuMAb HER2 by HIC. Results were also compared with the control samples stored in the dark at 5 °C for 2 week.

aggregate formation and deamidation of the protein as determined by native SEC and IEC (Table 3).

Mechanisms for Prevention of Methionine Oxidation by Antioxidants—Although methionine oxidation did not affect the activity of rhuMAb HER2, it may impact its immunogenicity or shelf-life. Potential methods to reduce temperature-induced methionine oxidation of rhuMAb HER2 were investigated to select inhibitors of oxidation and understand the mechanism of oxidation. As shown in Figure 4, rhuMAb HER2 in Formulation A containing added antioxidants, such as methionine, sodium thiosulfate, catalase, or platinum, did not oxidize after 2 weeks at 40 °C. In contrast, the control sample without antioxidant consisted of 52% oxidized Fc under the same storage conditions. Interestingly, removal of oxygen in the sample vials by repeated pulling vacuum and replacing with nitrogen was also effective in reducing rhuMAb HER2 oxidation. These results support the proposed mechanism that temperature-induced methionine oxidation of rhuMAb HER2 in the NaCl-containing Formulation A occurred by free radical formation in the presence of molecular oxygen. Methionine and thiosulfate can either inhibit free radical-induced oxidation by terminating the chain reaction or simply by competing with the methionine residues in rhuMAb HER2 for reaction with the free hydroxyl radicals. The free radical scavengers, catalase and platinum, also prevented methionine oxidation in rhuMAb HER2.



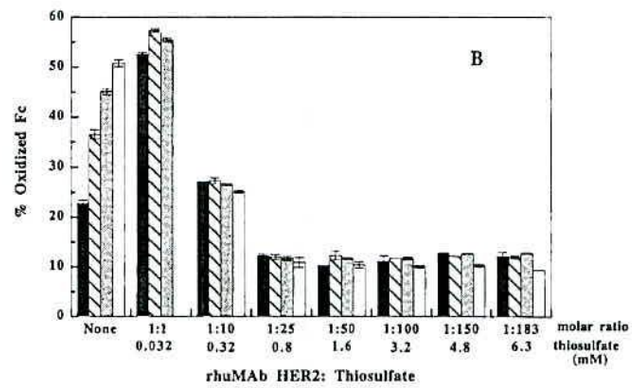


Figure 6—(A) Determination of the minimum effective level of methionine required to prevent oxidation of rhuMAb HER2. (B) Determination of the minimum effective level of sodium thiosulfate required to prevent oxidation of rhuMAb HER2. Methionine or thiosulfate was added to the rhuMAb HER2 Formulation A at different protein-to-antioxidant molar ratios before filling. Samples were stored at 40 °C for 1 week (black), 2 weeks (striped), 3 weeks (gray), and 4 weeks (white) and analyzed for oxidation of rhuMAb HER2 by HIC at the end of each storage period.

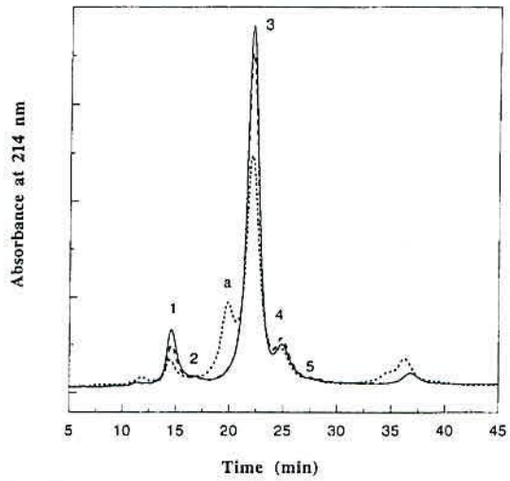


Figure 7—Interaction of sodium thiosulfate with rhuMAb HER2 detected by cation-exchange chromatography (IEC). After storage in a light box for 2 weeks, a degradation species (peak a) was observed in samples containing 6.3 mM thiosulfate (dotted line) as compared with the samples containing 3.5 mM methionine (dashed line) or no antioxidant (solid line). Peaks 1–5 are the five major isoforms of rhuMAb HER2 analyzed by IEC.

The effect of antioxidants on light-induced methionine oxidation in rhuMAb HER2 Formulation A was also studied (Figure 5). Unwrapped sample vials, containing either 6.3 mM sodium thiosulfate or 3.5 mM methionine (concentrations commonly used in parenteral pharmaceuticals), did not show an increase in oxidation when stored in a light box for 2 weeks. In contrast, oxidized Fc was increased to 48% for the unwrapped sample without antioxidants. These results suggest that methionine and sodium thiosulfate can be used for inhibiting photolytic oxidation in addition to temperature-induced oxidation of rhuMAb HER2. These antioxidants may prevent photolytic oxidation of rhuMAb HER2 by competing with methionine residues on the protein to react with molecular oxygen or singlet oxygen.

Different concentrations of methionine and thiosulfate were added to the rhuMAb HER2 Formulation A to determine the minimum effective level required to inhibit protein oxidation. Methionine at a final concentration of 0.175 mM, equivalent to a protein-to-methionine molar ratio of 1:5, was the minimum effective level for reduction of rhuMAb HER2 oxidation caused by temperature (Figure 6a). Thiosulfate at a minimum concentration of 0.8 mM, protein-to-thiosulfate molar ratio of 1:25, was required to inhibit rhuMAb HER2 oxidation in Formulation A (Figure 6b). Unlike methionine, thiosulfate reacted with rhuMAb HER2 as detected by cation-exchange chromatography (Figure 7). At a 1:100 molar ratio of protein-to-thiosulfate, an unknown degradation species (peak a) was observed in the sample. This peak may be an adduct between rhuMAb HER2 and thiosulfate yielding a new charged species. This species was more acidic than the native rhuMAb HER2 and eluted before the main peak of the native protein (peak 3). These data demonstrate that methionine is the preferred antioxidant for preventing rhuMAb HER2 oxidation.

Conclusions—Based on these studies, we conclude that temperature and light induce methionine oxidation of rhuMAb HER2. The rate of oxidation increases with temperature and is formulation dependent. The corrosion of stainless steel by sodium chloride present in rhuMAb HER2 liquid formulation to generate iron ions was the major catalyst for the protein oxidation at high temperatures. Temperature-induced oxidation of rhuMAb HER2 may occur by the formation of free radicals, and photo-induced oxidation may occur via singlet oxygen pathway. Methionine oxidation of rhuMAb HER2 either caused by temperature or light can be reduced by adding a stoichiometric amount of methionine or thiosulfate (antioxidants) to the formulation, but thiosulfate reacted with the protein. A protein-to-methionine molar ratio of 1:5 can inhibit rhuMAb HER2 oxidation.

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