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LUZ CLEMENCIA DE PÁEZ

ASUNTO

Radicación	07-88035
Trámite	11
Evento	379
Actuación	519
Oficio	
Folios	8

12131

Patente de Invención	☒			Patente de Modelo de Utilidad	☐
Vía Nacional	☐				
Vía PCT (Fase Nacional)	☒	Capítulo I	☐	Capítulo II	☒

No. Solicitud Internacional PCT/US2006/002837
Fecha de Presentación Internacional 27/Enero/2006

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

1. Documentos estudiados

Descripción:	Folios 134 a 226	28/Agosto/2007
Reivindicaciones:	Folios 227 a 241	28/Agosto/2007
	Folios 364 a 375	30/Marzo/2009
Dibujos:	Folios 243 a 253	28/Agosto/2007
Listado de Secuencias:	Folios 254 a 295	28/Agosto/2007
Prioridad:	US 60/648,631	28/Enero/2005

2. Análisis de la Prioridad

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

3. Modificaciones aceptadas

Se acepta el nuevo capítulo reivindicatorio (Fls. 364 a 375) porque no se amplía el objeto de la Solicitud.

4. Objeto de la invención

Título de la solicitud: "Formulación de anticuerpo anti A beta".

El problema planteado en esta solicitud consiste en la necesidad de suministrar una formulación que mantenga la estabilidad y actividad biológica de un polipéptido de unión Aβ y que sea adecuada para varias rutas de administración terapéuticas.

Para resolver este problema técnico la solicitud en estudio proporciona una formulación estable del anticuerpo monoclonal 3D6.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/395.

5. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 carece de concisión porque utiliza la expresión "una versión humanizada del anticuerpo monoclonal 3D6", lo cual dificulta determinar la extensión del objeto que se busca proteger. Se recuerda al solicitante que un polipéptido se define por su secuencia y un anticuerpo por la secuencia de las regiones variables de las cadenas ligera y pesada o la secuencia de sus 6 regiones determinantes de complementariedad (CDRs).

El capítulo reivindicatorio no es claro porque utiliza las expresiones "una versión humanizada del anticuerpo monoclonal 3D6", "anticuerpo 3D6 humanizado", "10D5", "266", "15C11", "9G8", "1C2", "2B1", "3A3", "6C6", lo cual no permite que la invención pueda ser comprendida y ejecutada por una persona capacitada en la materia técnica. Se requiere al solicitante presentar el Certificado de Depósito para los anticuerpos a los que se hace referencia el número de acceso ATCC.

El capítulo reivindicatorio utiliza la expresión "donde la formulación tiene un pH de (...)", que es un resultado a alcanzar y no define a la formulación puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales que contribuyen a la obtención de ese efecto, como los componentes y las proporciones de los mismos dentro de la formulación.

El capítulo reivindicatorio no es claro cuando utiliza el término "aproximadamente", el cual es impreciso y no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto.

Se requiere al Solicitante hacer las aclaraciones necesarias.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Enero 28 de 2005, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	US 6,750,324	Humanized and chimeric N-terminal amyloid beta-antibodies	15/Junio/2004
D2	US 2003/0202972	Protein formulation	30/Octubre/2003

D1 http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20040615&CC=US&NR=6750324B1&KC=B1 divulga una formulación farmacéutica que comprende un anticuerpo de unión Aβ 3D6 en una concentración de 5 mg/mL y un agente de amortiguación como histidina 50 mM (Reivindicación 1 y Columna 29, líneas 48-51).

D2 http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20031030&CC=US&NR=2003202972A1&KC=A1 divulga una formulación farmacéutica estable que comprende un anticuerpo humanizado en una concentración de 5-40 mg/mL (Párrafo 19), manitol en una concentración de 10-400 mM (0.2-7.3% p/v) (Párrafo 96) e histidina en una concentración de 1-20 mM (Párrafo 95) y metionina (Párrafo 100).

7. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La **reivindicación 1** consiste en una formulación estable que comprende:

a) una versión humanizada del anticuerpo monoclonal 3D6 (ATCC número de acceso PTA-5130) a una concentración de aproximadamente 1 mg/mL a aproximadamente 100 mg/mL;
b) histidina a una concentración de aproximadamente 0.1 mM a aproximadamente 25 mM; y
c) manitol en una concentración de aproximadamente 1% p/v a aproximadamente 10% p/v.

En donde la formulación tiene un pH de aproximadamente 5.5 a aproximadamente 6.5.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Una formulación estable que comprende: a) una versión humanizada del anticuerpo monoclonal 3D6 a una concentración de aproximadamente 1 mg/mL a aproximadamente 100 mg/mL;	Una formulación que comprende un anticuerpo humanizado de unión Aβ 3D6 en una concentración de 5 mg/mL (Columna 29, líneas 48-51)	Una formulación que comprende un anticuerpo humanizado en una concentración de 5-40 mg/mL (Párrafo 19)
b) histidina a una concentración de aproximadamente 0.1 mM a aproximadamente 25 mM; y	histidina en una concentración de 50 mM	histidina en una concentración de 1-20 mM (Párrafo 95)
c) manitol en una concentración de aproximadamente 1% p/v a aproximadamente 10% p/v	manitol (columna 30, línea 6)	manitol en una concentración de 10-400 mM (0.2-7.3% p/v) (Párrafo 96)

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D1 divulga una formulación farmacéutica que comprende un anticuerpo de unión Aβ en una concentración de 5 mg/mL y un agente de amortiguación como histidina 50 mM (Reivindicación 1 y Columna 29, líneas 48-51). El documento D1 también divulga que el anticuerpo se une específicamente a un epítopo dentro de los residuos 1-5 de Aβ, y que corresponde al anticuerpo 3D6 (Columna 61, líneas 20-40), el cual es del isotipo IgG1 (Columna 2, línea 55) y que está presente en una formulación junto con otros agentes farmacéuticamente aceptables y conocidos en el estado del arte (Columna 29, líneas 7-11), como el agente de tonicidad manitol (columna 30, línea 6) y el agente adyuvante polisorbato 80 (Columna 28, línea 31).

La diferencia entre la invención, definida en la reivindicación 1 y la formulación de D1 consiste en que comprende manitol en una concentración de 1% p/v a 10% p/v.

D2 divulga una formulación estable que comprende un anticuerpo humanizado en una concentración de 5-40 mg/mL (Párrafo 19), manitol en una concentración de 10-400 mM (0.2-7.3% p/v) (Párrafo 96) e histidina en una concentración de 1-20 mM (Párrafo 95) y metionina (Párrafo 100). D2 también enseña que la formulación puede comprender polisorbato 80 en una concentración de 0.005% a 0.05% (Párrafo 98), metionina (Párrafo 100) y sucrosa en una concentración de 10-400 mM (0.3-1.4% pv) (Párrafo 96).

La diferencia entre la invención definida en la reivindicación 1 y la formulación de D2 consiste en que comprende el anticuerpo monoclonal 3D6.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la formulación reclamada en esta solicitud porque no parece presentar una mayor estabilidad según lo establecido en la descripción.

El problema técnico objetivo de esta solicitud consiste en la necesidad de una formulación del anticuerpo monoclonal 3D6 alternativa a la ya conocida en D1.

Teniendo en cuenta que D1 ya divulgaba una formulación farmacéutica que comprende un anticuerpo de unión Aβ 3D6 en una concentración de 5 mg/mL y un agente de amortiguación como histidina 50 mM y que D2 enseña una formulación de un anticuerpo en una concentración de 5-40 mg/mL que comprende el lioprotector manitol en una concentración de 10-400 mM (0.2-7.3% p/v) e histidina en una concentración de 1-20 mM, la cual es estable durante el almacenamiento y hasta la administración al paciente, la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, optimizar de forma rutinaria la formulación de D1, para obtener una formulación estable del anticuerpo monoclonal 3D6.

De manera que la solución propuesta por esta solicitud en la reivindicación 1 y sus dependientes 2 – 15, es obvia y no tiene Nivel Inventivo.

La **reivindicación 16** consiste en una formulación estable que comprende:
(a) una versión humanizada del anticuerpo monoclonal 3D6 (ATCC número de acceso PTA-5130) a una concentración de 20 mg/mL, en donde el anticuerpo comprende una cadena liviana que comprende la secuencia de aminoácido SEQ ID NO:1 y una cadena pesada que comprende la secuencia de aminoácido SEQ ID NO:2;
(b) L-histidina a una concentración de 10 mM;
(c) D-manitol en una cantidad de 4% p/v.
(d) Metionina a una concentración de 10 mM; y
(e) Polisorbato 80 en una cantidad de 0.005% p/v,

en donde la formulación tiene un pH de 6.0.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Una formulación estable que comprende: (a) una versión humanizada del anticuerpo monoclonal 3D6 (ATCC número de acceso PTA-5130) a una concentración de 20 mg/mL, en donde el anticuerpo comprende una cadena liviana que comprende la secuencia de aminoácido SEQ ID	Una formulación que comprende un anticuerpo humanizado de unión Aβ 3D6 en una concentración de 5 mg/mL (Columna 29, líneas 48-51)	Una formulación que comprende un anticuerpo humanizado en una concentración de 5-40 mg/mL (Párrafo 19)

NO:1 y una cadena pesada que comprende la secuencia de aminoácido SEQ ID NO:2;		
(b) L-histidina a una concentración de 10 mM;	histidina en una concentración de 50 mM	histidina en una concentración de 1-20 mM (Párrafo 95)
(c) D-manitol en una cantidad de 4% p/v.	manitol (columna 30, línea 6)	manitol en una concentración de 10-400 mM (0.2-7.3% p/v) (Párrafo 96)
(d) metionina a una concentración de 10 mM; y	No se menciona	Metionina (Párrafo 100)
(e) polisorbato 80 en una cantidad de 0.005% p/v.	polisorbato 80 (Columna 28, línea 31).	polisorbato 80 en una concentración de 0.005% a 0.05% (Párrafo 98)

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D1 divulga una formulación farmacéutica que comprende un anticuerpo de unión Aβ en una concentración de 5 mg/mL y un agente de amortiguación como histidina 50 mM (Reivindicación 1 y Columna 29, líneas 48-51). El documento D1 también divulga que el anticuerpo se une específicamente a un epítopo dentro de los residuos 1-5 de Aβ, y que corresponde al anticuerpo 3D6 (Columna 61, líneas 20-40), el cual es del isotipo IgG1 (Columna 2, línea 55) y que está presente en una formulación junto con otros agentes farmacéuticamente aceptables y conocidos en el estado del arte (Columna 29, líneas 7-11), como el agente de tonicidad manitol (columna 30, línea 6) y el agente adyuvante polisorbato 80 (Columna 28, línea 31). D1 igualmente enseña una forma de dosis inyectable que comprende la formulación del anticuerpo 3D6 (Columna 29, líneas 33-38).

La diferencia entre la invención, definida en la reivindicación 16 y la formulación de D1 consiste en que comprende metionina y en la concentración del anticuerpo y de los excipientes histidina, manitol y polisorbato 80.

D2 divulga una formulación estable que comprende un anticuerpo humanizado en una concentración de 5-40 mg/mL (Párrafo 19), manitol en una concentración de 10-400 mM (0.2-7.3% p/v) (Párrafo 96) e histidina en una concentración de 1-20 mM (Párrafo 95) y metionina (Párrafo 100). D2 también enseña que la formulación puede comprender polisorbato 80 en una concentración de 0.005% a 0.05% (Párrafo 98), metionina (Párrafo 100) y sucrosa en una concentración de 10-400 mM (0.3-1.4% pv) (Párrafo 96). D2 igualmente divulga formas de dosis que comprenden la formulación del anticuerpo (Párrafos 112-113), artículos de manufactura que comprende un envase de vidrio que contiene la formulación liofilizada (Párrafo 118) y un método para preparar un producto farmacéutico que comprende las etapas de a)

liofilizar una mezcla del anticuerpo y un lioprotector, b) reconstituir la mezcla liofilizada en un diluente de manera que la formulación reconstituida es isotónica y estable (Párrafo 16).

La diferencia entre la invención definida en la reivindicación 16 y la formulación de D2 consiste en que comprende el anticuerpo monoclonal 3D6 y metionina 10 mM.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la formulación reclamada en esta solicitud porque no parece presentar una mayor estabilidad según lo establecido en la descripción.

El problema técnico objetivo de esta solicitud consiste en la necesidad de una formulación del anticuerpo monoclonal 3D6 alternativa a la ya conocida en D1.

Teniendo en cuenta que D1 ya divulgaba una formulación farmacéutica que comprende un anticuerpo de unión Aβ 3D6 en una concentración de 5 mg/mL y un agente de amortiguación como histidina 50 mM y que D2 enseña una formulación de un anticuerpo en una concentración de 5-40 mg/mL que comprende el lioprotector manitol en una concentración de 10-400 mM (0.2-7.3% p/v) e histidina en una concentración de 1-20 mM, la cual es estable durante el almacenamiento y hasta la administración al paciente, la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, optimizar de forma rutinaria la formulación de D1, más aun teniendo en cuenta que es bien conocido en el estado del arte que la metionina es un agente antioxidante utilizado para estabilizar formulaciones de anticuerpos[1], para obtener una formulación estable del anticuerpo monoclonal 3D6.

De manera que la solución propuesta por esta solicitud en la reivindicación 16 y las reivindicaciones 17 – 96, es obvia y no tiene Nivel Inventivo.

8. Conclusión

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

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US 6,750,324	1 – 96	Nivel Inventivo
D2	US 2003/0202972	1 – 96	Inventivo

1. X Lam, et al. Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2. Journal of Pharmaceutical Sciences, Vol. 86, No. 11, Noviembre 1997.

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

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta a este requerimiento y presentarla por escrito

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

JESÚS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha 13 JUL. 2012

Elaborado por: Juan David Moncaleano
Revisado por: Consuelo Leguizamón.

Antioxidants for Prevention of Methionine Oxidation in Recombinant Monoclonal Antibody HER2

XANTHE M. LAM[X], JANET Y. YANG, AND JEFFREY L. CLELAND

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.

S0022-3549(97)00143-3 CCC: $14.00

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

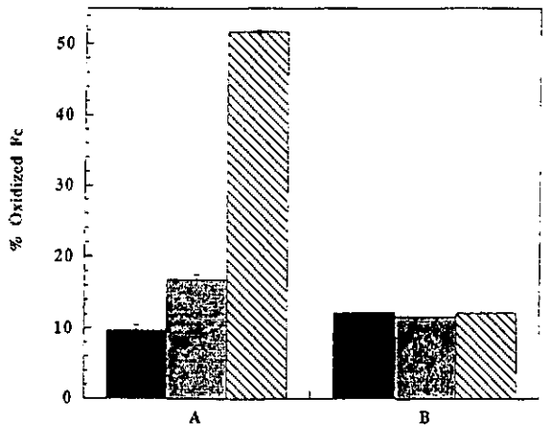


Figure 1—Effect of temperature on oxidation of rhuMAb HER2 in Formulations A and B. Samples were analyzed for methionine oxidation by HIC after 2 weeks of incubation at 5 °C (black), 30 °C (gray), and 40 °C (striped).

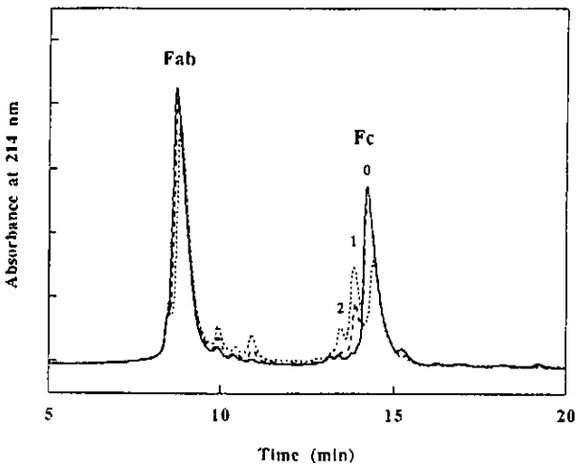


Figure 2—HIC chromatograms showing the effect of temperature on oxidation of the two methionine residues on the Fc domain of rhuMAb HER2 (Formulation A). Samples were incubated at 5 °C (solid line), 30 °C (dashed line), and 40 °C (dotted line) for 2 weeks. The three main peaks of the rhuMAb HER2 Fc domain 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 ($HO_2^•$), which can be terminated by reacting with the hydroxyl methionine free radical to form methionine sulfoxide, as shown in the following proposed mechanism:

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

Propagation
$$RO^• + R'\text{-}S\text{-}CH_3 \rightarrow R'\text{-}S^•_+\text{-}CH_3 + RO$$
$$R'\text{-}S^•_+\text{-}CH_3 + OH^- \rightarrow R\text{-}S^•(OH)\text{-}CH_3 \quad (2)$$
$$RO^• + O_2 + H_2O \rightarrow HO_2^• + ROOH$$

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

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

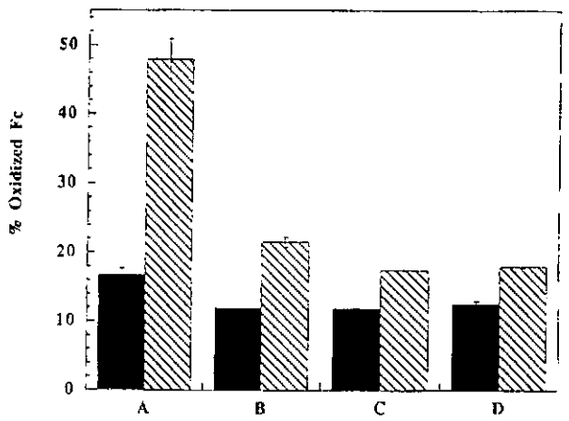


Figure 3—Effect of light on oxidation of rhuMAb HER2 in various formulations. Wrapped (black) and unwrapped (striped) samples were stored in a ligth box with light intensity of 20 000 lux for 2 weeks. The light box temperature was 27 °C. After light exposure, samples were analyzed for methionine oxidation 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	105 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.

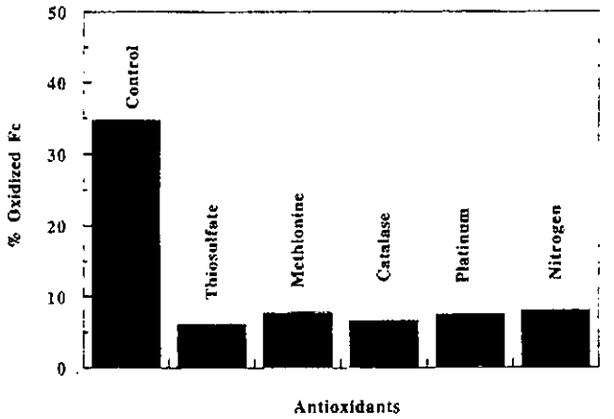


Figure 4—Methods to prevent temperature-induced oxidation of rhuMAb HER2 in Formulation A. Antioxidants were added to the formulation before filling into sample vials for incubation at 40 °C for 2 weeks. After incubation, samples were assayed by HIC to assess methionine oxidation. The amount of oxidation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

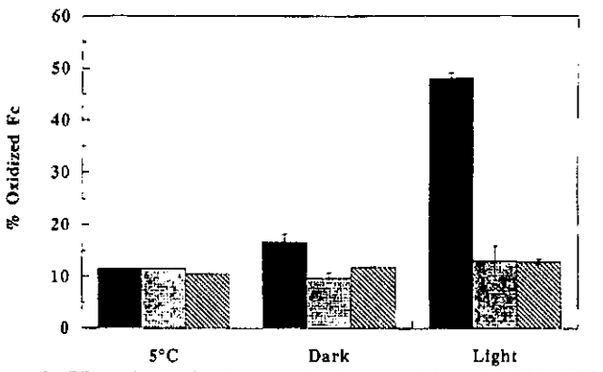
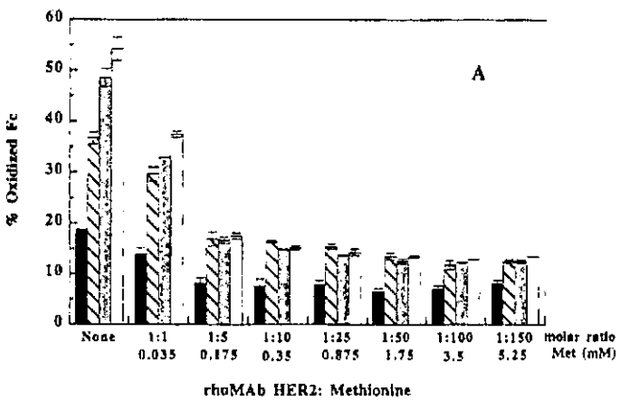


Figure 5—Effect of antioxidants on light-induced oxidation of rhuMAb HER2 Formulation A. Antioxidants were added to the formulation before filling into sample vials. Sample containing no antioxidant (black), 6.3 mM sodium thiosulfate (gray), and 3.5 mM methionine (striped) were stored wrapped (Dark) 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.



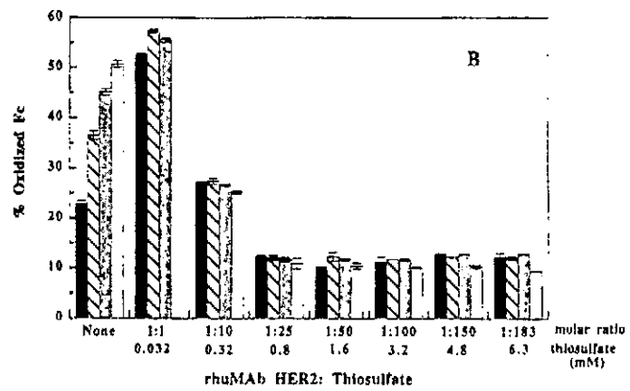


Figure 6—(A) Determination of the minimum effective level of methionine required to prevent oxidation of rhuMAb HER2. (B) Determination of the minimum effective level of sodium thiosulfate required to prevent oxidation of rhuMAb HER2. Methionine or thiosulfate was added to the rhuMAb HER2 Formulation 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.

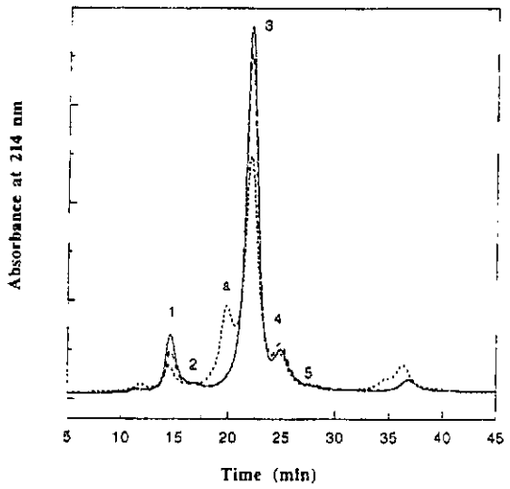


Figure 7—Interaction of sodium thiosulfate with rhuMAb HER2 detected by cation-exchange chromatography (IEC). After storage in a light box for 2 weeks, a degradation species (peak a) was observed in samples containing 6.3 mM thiosulfate (dotted line) as compared with the samples containing 3.5 mM methionine (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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