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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Bogotá D.C., 19 de agosto de 2014

Señores
DIVISION DE NUEVAS CREACIONES
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Ciudad

REFERENCIA: Expediente No. 13 – 009.151

TÍTULO: “UN NUEVO CONJUGADO DE GRANULOCITO COLONIA-
ESTIMULANTE FACTOR (G-CSF) CON POLIETILENO”

SOLICITANTE: CLOSED JOINT STOCK COMPANY BIOCAD

TRÁMITE: Respuesta a Concepto Técnico notificado mediante Oficio No.
6.309 del 3 de junio de 2014.

JAIME EDUARDO DELGADO VILLEGAS, en mi condición de apoderado de la CLOSED JOINT STOCK COMPANY BIOCAD, dentro del término legal, presento respuesta a Concepto Técnico notificado mediante Oficio No. 6.309 del 3 de junio de 2014.

1. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El Examinador Técnico de Patentes objeto el Capítulo Reivindicatorio porque:

“La reivindicación 1 no es clara porque a pesar de que se encabeza como “Un factor estimulante de colonias de granulocitos humanos”, el objeto de la solicitud comprende un conjugado de este junto a

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monometoxipolietilen glicol. Por lo tanto se le sugiere a la solicitante encabezar la reivindicación por la expresión “Un conjugado (...)”.

“La reivindicación 1 no es concisa porque la expresión “actividad de G-CSF” da la posibilidad de un sinnúmero de moléculas que presenten dicha actividad. Se sugiere restringir la solicitud a una razonable generalización del tipo de conjugados que ha sido probado, es decir que se encuentran debidamente sustentados”.

“La reivindicación 1 no se encuentra sustentada en la descripción porque el índice m no tiene sustento para todos los valores mayores o igual a 4. Únicamente se encuentra sustento en el capítulo descriptivo cuando m es 4.

“La reivindicación 1 no es clara porque reclama una proteína pero no la caracteriza por su secuencia”.

“La reivindicación 1 no es clara porque no caracteriza la proteína de manera adecuada. Se recuerda a la solicitante que un péptido debe caracterizarse por su secuencia de aminoácidos haciendo referencia a la correspondiente SEQ ID NO. Y adicionalmente en caso de que la proteína presente modificaciones tendrán que ser nombradas”.

“La reivindicación 7 no es clara porque a pesar de que se encabeza con el término composición, no la caracteriza de forma adecuada. Se le aclara a la solicitante que una composición está definida en términos de ingredientes con su respectiva proporción...”.

“La reivindicación 10 no es clara porque caracteriza un contenedor con la composición que almacena, lo cual es incorrecto teniendo en cuenta que un contenedor se caracteriza por su composición y forma”.

“Las reivindicaciones 10 y 11 no son claras porque presentan una falsa dependencia con la reivindicación 6, puesto que caracterizan un recipiente con la composición que contiene lo cual es incorrecto. Se sugiere hacer las correcciones necesarias”.

“La reivindicación 11 no es clara porque describe un contenedor farmacéutico, con características inherentes de la composición

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farmacéutica contenida. Se le llama la atención a la solicitante que un contenedor farmacéutico no se debe caracterizar por su contenido o viceversa, sino por sus características técnicas estructurales y material de fabricación. Se debe omitir la mención de estos contenedores, ya que no se establece diferencia con el estado de la técnica ni el campo técnico de esta solicitud”.

“La reivindicación 11 no es clara ni se encuentra sustentada en la descripción, porque incluye elementos de métodos de tratamiento y adicionalmente reclama un Kit que no se encuentra sustentado en el capítulo descriptivo. Adicionalmente se le aclara a la solicitante que un kit está definido por dos unidades funcionales independientes. Se sugiere eliminar o hacer correcciones necesarias”.

R/: Nuestro poderdante de acuerdo a lo expresado por el Examinador Técnico de Patentes, procedió a modificar el capítulo reivindicatorio, teniendo en cuenta:

Las Reivindicaciones relacionadas a la composición farmacéutica se **modificaron incorporando la característica esencial**, en particular, indicando la composición definida (proporción definida de los componentes contenidos en la composición farmacéutica) a partir de la descripción (la composición se puede encontrar la descripción, ejemplo 17).

La Reivindicación 1 se modifica donde comprende el inicio de la definición “un conjugado...”.

El índice se definió como **m es igual a 4**, el cual está soportado en la descripción y la característica “NaH-G-CSF” se define mediante la indicación de la secuencia de aminoácidos correspondiente a G-CSF usado para la pegilación. Además, la secuencia de aminoácidos relacionada con G-CCSF utilizado para la preparación

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de los conjugados de la Reivindicación 1 que están incorporados en la descripción
(páginas 4, 6-8)

Descripción (Art 28 D 486)

El Examinador Técnico de Patentes objeto la Descripción de la invención porque:

“La descripción no es clara porque no define de manera adecuada el péptido que se une al polímero, adicionalmente se nombra el péptido estimulador de factor de crecimiento de colonias G-CSF y no se menciona su secuencia proteica, lo cuál no permite determinar si existen variaciones o corresponde a la secuencia natural”.

“Se requiere al solicitante hacer las aclaraciones necesarias”.

2. Unidad de invención (Art 25 D 486)

El Examinador objeta la **unidad de invención** porque:

“La solicitud no cumple con el requisito de unidad de invención porque se refiere a 2, grupos inventivos que se encuentran definidos en dos campos técnicos diferentes”.

“Los grupos inventivos están definidos por las siguientes características esenciales:

1° Conjugado de conjugado del factor granulocito estimulante - polietilen glicol.

2° Un contenedor

Se sugiere presentar las solicitudes divisionales que considere necesarias, o limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones”.

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R/: Nuestro poderdante de acuerdo a lo expresado por el Examinador Técnico de Patentes, procedió a **eliminar las Reivindicaciones 9 a 11** del capítulo reivindicatorio, relacionadas con el Contenedor y se excluyen del objeto de la invención.

Por lo expresado anteriormente, el solicitante pone a consideración del Examinador Técnico de Patentes un **NUEVO CAPITULO REIVINDICATORIO**, el cual consta de **seis (6) Nuevas Reivindicaciones** en un folio.

3. DEL ANÁLISIS DE PATENTABILIDAD.

Nivel Inventivo (art.16 D 486)

El Examinador objeta el **nivel inventivo** para las reivindicaciones 1 a 12 con los documentos **D1 (US5824784)**, **D2 (WO2004083242)**, **D3 (US5252714)** y **D4 (Experimental Hematology 27 (1999) 425–432)** porque:

“(…)”

“Nivel inventivo D1 y D2

“La diferencia entre la invención, definida en la reivindicación 1 y el conjugado del documento D1 corresponde al peso molecular del derivado de PEG”.

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“El efecto que logra el conjugar un derivado de PEG de mayor peso molecular al factor estimulante de colonias de granulocitos es favorecer la estabilidad del conjugado y mejorar los parámetros farmacocinéticos”.

“(…)”

“En consecuencia, la persona del oficio normalmente versada en la materia, incluiría polímeros de PEG de mayor tamaño del documento D2 en los conjugados divulgados en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia”.

“Conclusión:

La reivindicación 1 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18)”.

“Puesto que las reivindicaciones 2-12 no mencionan características adicionales se considera que no tienen nivel inventivo”.

“Nivel inventivo D4 y D3

*“La diferencia entre la invención, definida en la reivindicación 1 y el conjugado de PEG- del factor estimulante de colonias de granulocitos del documento D4
corresponde al espaciador que enlaza la proteína con el polímero”.*

“El efecto que logra el conjugar un derivado de PEG con una proteína por medio de un agente enlazante de 4 átomos de carbono no evidencia un efecto técnico inesperado”.

“En consecuencia, la persona del oficio normalmente versada en la materia, intercambiaría los espaciadores del documento D3 en los espaciadores de los conjugados divulgados en el documento D4 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia”.

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“Conclusión:

La reivindicación 1 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18)”.

“Puesto que las reivindicaciones 2-12 no mencionan características adicionales se considera que no tienen nivel inventivo”.

R/: Con relación a las objeciones del Examinador de Patentes con respecto a la falta de **Nivel inventivo** de la invención en estudio, el solicitante desea resaltar lo siguiente:

Con relación a los documentos **D1** y **D2**:

El Examinador menciona que el documento **D1** revela un método para preparar un conjugado que consta de G-CSF y PEG de peso molecular de 6.000 a 25.000 Da (columna 13, Ejemplo 1, punto “B”) mientras que el documento **D2** describe los conjugados de PEG de G-CSF con peso molecular de PEG hasta 100 kDa.

De acuerdo con el documento **D1**, el método para preparar G-CSF pegilado incluye la conjugación de rh-G-CSF con ésteres N-hidroxi y succinimidil de carboximetil metoxi polietilen glicol a pH 8,0, y después de agitar la mezcla de reacción consistía principalmente en tres formas de rh-G-CSF monopegilado, algunos productos secundarios de la reacción rh-G-CSF

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dipegilado, rh-G-CSF no modificado y producto (N-hidroxi succinimida).

Como se conoce de la técnica anterior en química, el uso de diferentes reactivos iniciales bajo diferentes condiciones conduce a la preparación de diferentes productos finales. Así, el conjugado del documento **D1** tiene otro grupo de enlace entre PEG y G-CSF. Además, la pegilación en el documento **D1** se realizó bajo otra condición (pH, tiempo de agitación y proceso de aislamiento) **diferente a la solicitud de patente de invención de nuestro poderdante**, que da como resultado la preparación de la mezcla de isómeros posicionales (documento **D1**). Esto está soportado por la Tabla 1 y la Figura 1 y 1A, donde se indican los productos relativos de G-CSF pegilado con N-terminal en el documento **D1**. Por lo tanto, **el método para preparar y los conjugados preparados son diferentes entre sí**.

Además, con base en la descripción (página 18, Tabla 5 de la presente solicitud de patente de invención en estudio), **el PEG-G-CSF conjugado reivindicado en la solicitud en estudio tiene unas características mejores** que las de los conjugados del documento **D1**, donde la actividad del G-CSF pegilado es menor

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que la del G-CSF no modificado (Figura 4) y la estabilidad durante el almacenamiento es mucho menor que la del conjugado de PEG-G-CSF de la presente solicitud de patente.

La característica de la actividad específica de 84% y más con base en la actividad de G-CSF no modificado no se puede predecir con base en el documento D1, en particular la Figura 9 como parámetro de la actividad específica no se puede calcular con base en los datos de la técnica presente.

Las características clave que distinguen la invención reivindicada en el documento **D1** son las siguientes:

1. El documento **D1** revela la preparación de la composición de G-CSF modificado clínicamente N-terminal, opcionalmente en un portador, donde hasta el 95% del PEG-G-CSF conjugado está contenido en la composición mencionada (ver el documento **D1**, reivindicaciones 1 y 6-7) o una mezcla de isómeros posicionales (ver el documento **D1** Fig. 2, Fig. 5A, 5B, Tabla 1 en la página 28).

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Mientras tanto, la **invención reivindicada** brinda un **conjugado puro de G-CSF que tiene adjunción de PEG en metionima N-terminal solamente** y PEG que tiene peso molecular de 30 a 40 kDa.

2. El documento **D1** describe la preparación del conjugado PEG-G-CSF usando el propionaldehído (ver en el documento **D1** el diagrama en la página 17, página 20: “el polietilenglicol propionaldehído...es ventajoso por su estabilidad en agua”), mientras que la **invención reivindicada se relaciona con el conjugado preparado mediante Metoxi PEG-Butiraldehído (mPEG-ButyrALD)** (ver página a 8, Ejemplo 2, WO2012021088). En este caso la característica distintiva es el índice m: para el documento **D1** m es 3 (para propionaldehído), mientras que el **conjugado reivindicado en la presente patente de invención de nuestro poderdante tiene m de 4**.

Como se conoce en el estado de la técnica anterior, el proceso de conjugación que usa propionaldehído y el butiraldehído son diferentes entre sí en condiciones y puede dar como resultado la preparación de diferentes conjugados.

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Además, el derivado MPEG butiraldehído tiene los grupos CH_2 – adicionales, lo que significa que este aldehído tiene mayor lipofilicidad, otra estructura espacial, que puede conducir a la imposibilidad de preparar el conjugado reivindicado en la solicitud si se usa el método de preparación del documento **D1**.

Además, las formulas estructurales de los conjugados preparados de acuerdo con el documento **D1** (Ejemplo 2) y los de la presente solicitud de patente de invención reivindicada son diferentes, por lo cual las propiedades tales como actividad biológica, características químicas y físicas también son diferentes.

Así con base en lo anterior a medida que el butiraldehído y el propionaldehído tienen diferente estructura y propiedades químicas, los preparados conjugados son también diferentes, con base en el documento **D1** una persona experta en la materia no puede predecir la preparación exitosa de los conjugados usando el PEG-butiraldehído y que estos conjugados serán estables y biológicamente activos.

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3. El documento **D1** revela conjugados que tienen PEG de peso molecular (ver el Ejemplo 1 y 2, Tabla 4 de la página 39) de 6, 12, 20 y 25 kDa mientras que **el conjugado reivindicado en la presente solicitud de patente de nuestro poderdante incluye el PEG de peso molecular de 30 a 40 kDa.**

Debido a que las propiedades fisicoquímicas (es decir, viscosidad, solubilidad, densidad y otras) del PEG es dependiente del peso molecular del mismo, las condiciones de preparación son diferentes para los métodos que usan PEG de menor peso molecular (6 – 25 kDa) y de mayor peso molecular (30 – 40 kDa). Con base en lo anterior, se puede concluir que **el conjugado reivindicado de la presente solicitud de patente de invención** difiere del conjugado descrito en el documento **D1**.

La conclusión de que cuanto mayor sea el peso molecular de PEG tanto mayor será el conteo de glóbulos blancos logrado y no se puede realizar completamente con base en la Figura 9 del documento **D1** ya que el peso molecular de PEG no se puede

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aumentar sin disminuir la solubilidad del conjugado PEG-G-CSF o su toxicidad al administrarlo a un organismo. Además, la preparación del conjugado que tiene PEG de mayor peso molecular se asocia con problemas de acople ya que las unidades de PEG pueden obstruir estéricamente fijándose al aminoácidos de G-CSF. Igualmente, como se puede observar de los **ejemplos de la presente solicitud** (ver Tabla 5, página 18; Tabla 4, página 16, WO2012021088), **el conjugado reivindicado tiene una mejor actividad y mayor estabilidad en almacenamiento** que el conocido del estado de la técnica anterior.

Con respecto al documento **D2** este revela los conjugados de PEG CSF donde el peso molecular de PEG puede ser hasta de 100 kDa. Sin embargo, el G-CSF mencionado en el documento **D2** es derivado PEG-hG-CSF que incluye una sustitución de aminoácidos en la posición 17th de modo que este derivado no puede tener metionina extra terminal (página 5, líneas 18-24) así **las propiedades físicas y químicas de este derivado son diferentes a las de G-CSF simple o como en la presente solicitud de patente de invención en estudio.** Basado en lo

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anterior, se puede concluir que el G-CSF tiene otra secuencia de aminoácidos que el G-CSF en la **presente solicitud de patente de invención de nuestro poderdante** y con todo, los inventores **no indicaron la secuencia de aminoácidos para el derivado G-CSF y este derivado, difiere del revelado en la presente invención en estudio**, ya que este derivado tiene el reemplazo la posición 17 con serina y **no tiene metionina terminal como G-CSF en la solicitud de patente de invención en estudio**. Como se conoce en el estado de la técnica anterior, el reemplazo y la diferencia con la secuencia de aminoácidos en las proteínas pueden llevar a diferentes propiedades de las proteínas. Así, el otro aminoácido terminal puede ser inútil para la conjugación de PEG, **la proteína puede tener diferente punto isoelectrico o estructura espacial y no puede aplicarse un método para preparar un conjugado PEG como se revela en la presente solicitud de patente de invención de nuestro poderdante**.

Además, el documento **D2** (Ejemplo 1: Medición de actividad in vivo, tabla 1 indica que los conjugados PEG-G-CSF tienen menor actividad que el G-CSF no modificado. Los inventores concluyeron con base en los resultados obtenidos (página 16,

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líneas 1-5) que el mayor peso molecular de PEG ligado a la menor actividad de G-CSF in vivo.

Con base en lo mencionado anteriormente, con los documentos D1 y D2 una persona experta en la técnica no puede predecir que los conjugados de G-CSF y PEG de peso molecular 30 a 40 kDa puedan poseer más actividad que los conjugados de la técnica anterior.

Por otro lado, el Examinador señala que el documento **D1** en combinación con el documento **D2** revelan los conjugados de G-CSF y PEG, un método para preparar y purificar estos conjugados. Sin embargo, como ya se ha mencionado antes, las proteínas y conjugados revelados en los documentos **D1** y **D2** tienen estructura diferente a la del conjugado de la **presente solicitud de patente de invención de nuestro poderdante**, y el hecho de que el conjugado PEG-G-CSF de la reivindicación 1 **tenga mejores propiedades tales como terapéuticas, estabilidad, facilidad de preparación y otras no es evidente para un experto en la materia técnica.**

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Por otra parte, teniendo en cuenta que el peso molecular de PEG tiene efecto sobre sus propiedades fisicoquímicas tales como viscosidad, solubilidad, aceptabilidad terapéutica y no toxicidad (ver, por ejemplo, el fragmento de la Farmacopea Japonesa: **Journal of Polymer Science: Vol XLII**, páginas 195-202 (1960) adjunta; **Polymer Vol 38 No. 12**, pp 2885 – 2891, 1997 adjunta), **la persona experta en la materia no puede esperar que los conjugados de G-CSF con PEG de peso molecular 30 y 40 kDa tengan unas características terapéuticas mejores a los de conjugados con PEG de 6 a 25 kDa.**

Adicionalmente, una preparación de conjugado que tenga el PEG de mayor peso molecular se asocia con problemas en el acople ya que las unidades de PEG pueden obstruir estéricamente el acople al aminoácido de G-CSF o la viscosidad de una mezcla de reacción no es la misma que la velocidad de la mezcla de reacción que comprende PEG de MW de 6-20 kDa de modo que **una persona experta en la técnica no predice la posibilidad de preparación de conjugado PEG-G-CSF que tiene peso molecular de PEG de 30 a 40 kDa.** Las unidades de PEG en la mezcla de reacción que tienen mayor viscosidad pueden construir un sistema espacial que no permite la difusión de un iniciador o

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una proteína para la conjugación. Por lo tanto, con base en la descripción de los documentos **D1 y D2** la persona experta en la materia **no puede predecir la posibilidad de la preparación en el conjugado reivindicado en la presente solicitud de patente de invención ni el fenómeno de que los conjugados de PEG-G-CSF con peso molecular de 30 1 40 kDa según lo reivindicado en la reivindicación 1 de la invención en estudio, serán más activos y más estables que los anteriores.**

Por lo tanto, el objeto de la invención revelado en la presente solicitud de patente de invención implica NIVEL INVENTIVO y puede considerarse no evidente con respecto a los documentos D1 y D2 y es susceptible de patentabilidad.

Con respecto a los documentos **D3 y D4:**

El Examinador indica que el documento **D4** sustenta que el número de neutrófilos alcanzó un máximo incremento al aumentar el tamaño de PEG. Sin embargo, el documento **D4** revela la investigación sobre conjugados PEG preparados con una muteína recombinante de factor estimulador de colonias de granulocitos

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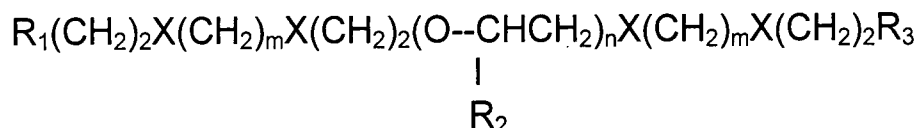
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(nartograstim [NTG], KW-2228). Naarogrstim es una modificación de G-CSF (ver **Suplemento 1 de la Farmacopea japonesa**, Vol 75, No.9 (May 1) 1990: pp 1788-1793 y no puede considerarse como nativo G-CSF, ya que estas proteínas tienen diferentes estructuras de aminoácidos y diferente actividad biológica y propiedades fisicoquímicas.

El documento **D3** revela los cojugados de la siguiente formula general,



Donde R₁, R₂, R₃ y R₄ se definen en la reivindicación 1 (Columna6) que puede usarse para preparar los conjugados PEG.

Sin embargo, los Ejemplos para pegilación de anticuerpos se presentan (Columna 5 y 6), indicando que el grupo de lisina se usan para la fijación (Columna 5, líneas 66 – 68). La **pegilación de G-CSF** de acuerdo con la **presente solicitud de patente de invención** está sobre la metionina N-terminal de modo que este

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método de preparación no puede usarse para pegilación de G-CSF.

Como se puede concluir de lo anterior con base en la combinación de los documentos D3 y D4 el método para la preparación del conjugado de PEG-G-CSE según lo reivindicado en la reivindicación 1 de la presente solicitud de patente de invención y especialmente este conjugado será activo en comparación con los conocidos en la técnica y no son evidentes para una persona experta en la técnica. Basado en estos argumentos, el solicitante encuentra que la invención objeto de las reivindicaciones modificadas presentadas implican NIVEL INVENTIVO Y PUEDEN SER PATENTADAS.

En conclusión, de acuerdo a lo expresado anteriormente, queda claramente demostrado que existen diferencias sustanciales entre los Conjugados que se describen en los documentos D1, D2, D3 y D4 mencionados, con respecto al Conjugado de granulocito colonia-estimulante factor (G-CSF) con polietileno que se desea proteger en la presente solicitud de patente de invención de nuestro poderdante; además, el solicitante pone a consideración del Despacho UN NUEVO CAPÍTULO REIVINDICATORIO el cual consta de seis (6) nuevas reivindicaciones en un (1) folio, el cual cumple con los requisitos de NOVEDAD, NIVEL INVENTIVO Y APLICACIÓN INDUSTRIAL exigidos por la Decisión 486 del Acuerdo de la Comisión de la Comunidad Andina y se esta dando respuesta al Concepto Técnico notificado mediante Oficio No. 6.309 del 3 de junio de 2014.

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Por todo lo anteriormente mencionado, solicito respetuosamente se proceda a **CONCEDER** la Patente de Invención titulada: **“UN NUEVO CONJUGADO DE GRANULOCITO COLONIA-ESTIMULANTE FACTOR (G-CSF) CON POLIETILENO”** a nombre de la sociedad **CLOSED JOINT STOCK COMPANY BIOCAD**, para las Nuevas Reivindicaciones 1 a 6 inclusive.

Atentamente,


JAIME EDUARDO DELGADO VILLEGAS
C.C. No. 19.270.955 Bogotá
T.P. No. 43.308 C.S.J.

Anexos:

Nuevas Reivindicaciones (6) en un folio.

Farmacopea Japonesa:

Journal of Polymer Science: Vol XLII,

Polymer Vol 38 No. 12.

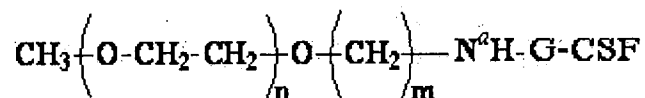
Suplemento 1 de la Farmacopea japonesa, Vol 75, No.9.

In Vitro and In Vivo Hematopoietic Effect of Mutant Human

Granulocyte Colony-Stimulating Factor (nartograstim [NTG], KW-2228)

NUEVAS REIVINDICACIONES

1. Un factor estimulante de colonias de granulocitos humanos conjugado con monometoxipolietilen glicol de fórmula (I) donde el sitio de conjugación del monometoxipolietilen glicol es la metionina N-terminal del factor estimulante de colonias de granulocitos humanos (G-CSF):



(I)

donde:

n – es un número entero de 681 a 1 000;

m – es 4;

N^aH-G-CSF - polipéptido que tiene la secuencia de aminoácidos de SEQ ID: 1.

2. Un conjugado de acuerdo con la reivindicación 1, donde el peso molecular promedio del polietilen glicol es de 30 a 40 kDa.
3. Un conjugado de acuerdo con la reivindicación 2, donde el peso molecular del polietilen glicol es 30 kDa.
4. Una composición farmacéutica, que tiene la actividad del factor estimulante de colonias de granulocitos, que comprende:
el conjugado de fórmula general (I) según la reivindicación 1, - 0,6 - 3,0 mg
excipientes:

Polisorbato	20 - 0,04 mg
Manitol	- 50 mg
Acetato trihidrato de sodio	- 0,23 mg
Acido acético glacial	hasta pH 4,0
Agua para inyección	hasta 1 ml.
5. Una composición farmacéutica según la reivindicación 4, en el que el valor de pH es de 3 a 5.
6. Una composición farmacéutica según la reivindicación 4, en el que el valor de pH es principalmente 4.

Viscosity Relationship in Solutions of Polyethylene Glycols

D. K. THOMAS* and A. CHARLESBY, *Physics Department, Royal Military College of Science, Shrivenham, Wilts, England*

INTRODUCTION

One property characteristic of polymers is their high viscosity in solution, and a great deal of experimental work has been done on such systems. Whereas most experiments of this nature are conducted on very high molecular weight polymers, this study is concerned with relationships between molecular weight, concentration, and viscosity at both relatively high and low molecular weights. In addition the measurements have been carried out in good and bad solvents.

The theoretical work of Einstein¹ in 1906 was the first rigorous hydrodynamic analysis of the viscosity of colloidal solutions, and for the ideal case of suspensions of rigid spheres of uniform size, in which the radius r of the particles is small with respect to the mean distance between them, he showed that the viscosity of the suspension should be given by the equation:

$$\eta/\eta_0 = 1 + 2.5 V_f \quad (1)$$

or

$$\eta_{sp} = (\eta - \eta_0)/\eta_0 = 2.5 V_f$$

η_{sp} being the specific viscosity, η_0 the viscosity of the solvent, and V_f the volume fraction of colloidal material. This equation represents the limiting case for infinitely small changes in viscosity; for moderately large changes it should give too small values for the relative viscosity ($= \eta/\eta_0$). From this relationship it appears that the specific viscosity of a dilute suspension is dependent only on the volume concentration and is thus entirely independent of particle size, temperature, or viscosity of the solvent. In general, high polymers in solution deviate from spherical form, and the viscosities of their solutions are many times greater than would be predicted by eq. (1). In addition, the theoretical treatment assumed no interaction between solute molecules. This may be a sufficiently good approximation at high dilution but will certainly break down as the concentration increases. The inclusion of solute interaction effects in the theoretical treatment for rigid spherical particles led to a modification of the Einstein equation by Guth and Gold:²

$$\eta_{sp} = 2.5 V_f + 14.1 V_f^2 \quad (2)$$

* Civil Service Commission Senior Research Fellow.

This equation is found to hold to higher concentrations than eq. (1) but is still of rather limited applicability. Relationships between viscosity and concentration have been developed theoretically for particles of shapes other than spherical, the case of soft as distinct from rigid spheres has been considered, as have those of rigid, rodlike particles³⁻⁶ and rigid disks.⁷ These latter cases lead to expressions relating the viscosity to the concentration and axial ratio of the solute molecules.

For high polymers, in practice, it is found that the variation of η_{sp}/c with c can often be expressed by an equation of the form

$$\eta_{sp}/c = [\eta] + a_2c \quad (3)$$

The quantity $[\eta]$, the intrinsic viscosity, is a measure of the viscosity behavior at infinite dilution, and the second term reflects the interaction amongst solute molecules. In many cases for not too high concentrations it has been found that $a_2 = k'[\eta]^2$, where k' is the Huggins constant, which varies with the nature of the polymer and solvent but is independent of the molecular weight within a particular polymer series. The value of k' is often found to be between 0.3 and 0.4 for linear polymers in good solvents. Thus

$$\eta_{sp}/c = [\eta] + k'[\eta]^2c \quad (4)$$

Most other relationships which have been proposed to account for the variation of the viscosity of polymer solutions with concentration can be reduced to a form closely approximating to this equation.

Over a certain range of concentration, and in poor solvents, the dissolved polymer should tend towards a more spherical configuration to which the Einstein or Guth and Gold relationships apply. In this paper the experimental results are compared with these relationships and with those deduced for other configurations.

EXPERIMENTAL

Viscosity measurements were made with an Ostwald viscometer at a temperature of $35 \pm 0.02^\circ\text{C}$. Union Carbide polyethylene glycols, having molecular weights of 400, 1,000, 1,500, and 4,000 were used. These molecular weights had been determined by endgroup analysis, and in any one sample the variation from the mean was $\pm 5\%$.

The solvents used were water and toluene, the latter representing a poor solvent for polyethylene glycols, and all solutions were made up gravimetrically.

RESULTS

The results for aqueous and toluene solutions are shown in Figure 1 and 2, respectively. These lead to the values for intrinsic viscosity shown in Table I.

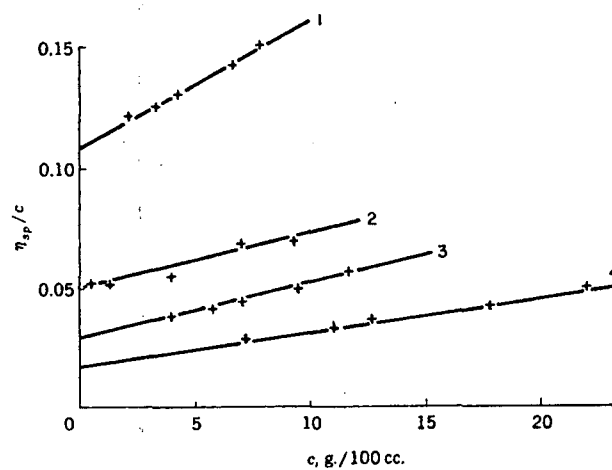


Fig. 1. Plot of η_{sp}/c vs. c in aqueous solution for (1) Carbowax 4000; (2) Carbowax 1500; (3) Carbowax 1000; (4) Carbowax 400.

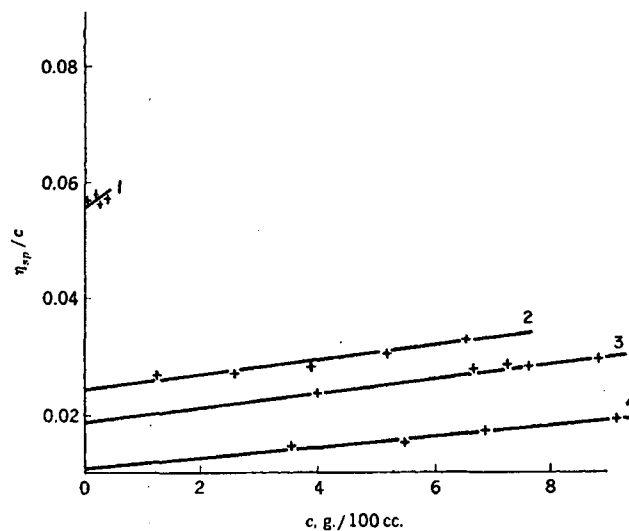


Fig. 2. Plot of η_{sp}/c vs. c in toluene for (1) Carbowax 4000; (2) Carbowax 1500; (3) Carbowax 1000; (4) Carbowax 400.

TABLE I
Values for Intrinsic Viscosities and Huggins Constants
of Polyethylene Glycols in Water and Toluene

Mol. wt.	[η]		k'	
	Water	Toluene	Water	Toluene
400	0.0167	0.0105	4.95	8.16
1000	0.0298	0.0186	2.42	3.41
1500	0.0501	0.0243	0.93	2.06
4000	0.108	0.0546	0.44	2.3

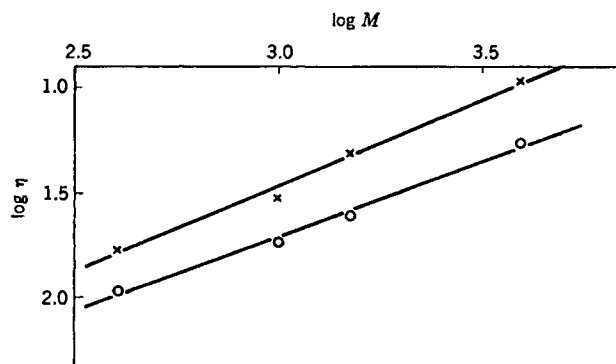


Fig. 3. Plot of $\log M$ vs. $\log \eta$ of polyethylene glycols in (X) aqueous solution; (O) in toluene solution.

Figure 3 shows $\log [\eta]$ as a function of $\log M$. In both cases a linear relationship is obtained, hence $[\eta] = KM^\alpha$. For aqueous solutions, $K = 1.66 \times 10^{-4}$ and $\alpha = 0.82$, while, for toluene solutions, $K = 1.45 \times 10^{-4}$ and $\alpha = 0.70$.

As would be anticipated, for a flexible linear polymer the intrinsic viscosity is higher in a good solvent (water) than in a poor one (toluene), showing that it has taken up a more extended form in the former.

The values of the Huggins constant, k' of eq. (4), are given in Table I.

DISCUSSION

As mentioned previously, many polymer systems show specific viscosities which vary with concentration according to the Huggins equation:⁸

$$\eta_{sp}/c = [\eta] + k'[\eta]^2c$$

This equation is deemed applicable to linear polymers in good solvents only, and would be expected to apply to the aqueous rather than to the toluene solutions. Values of k' are usually found in the range 0.3 to 0.5, but Table I reveals that only for aqueous polyethylene glycol with a molecular weight of 4000 is a value obtained within these limits. It may be that only when the molecular weight reaches this value is one considering a high polymer in the sense required by the Huggins equation. Previous applications, in which the k' values fall between 0.3 and 0.5, have been made in solutions of genuine high polymers having molecular weights of many thousands.

The quantity c in the Huggins equation is the concentration expressed as grams of polymer per 100 cubic centimeters of solvent. If this were expressed as a volume fraction V_f , eq. (4) would become

$$\eta_{sp}/V_f 100\rho_p = [\eta] + k'[\eta]^2 100\rho_p V_f$$

or

$$\eta_{sp} = [\eta] 100\rho_p V_f + k'[\eta]^2 \times 10^4 \rho_p^2 V_f^2 \quad (5)$$

By comparison with the Einstein equation it follows that

$$[\eta] = 2.5/100\rho_p$$

where ρ_p is the density of the polymer.

Hence one would anticipate that, regardless of the nature of the solvent, polyethylene glycols of density 1.1 should have intrinsic viscosities of about 0.022. Furthermore, by making a similar substitution for V_f in the equation of Guth and Gold, we obtain:

$$\eta_{sp}/c = (2.5/100\rho_p) + 14.1(1/100\rho_p)^2c$$

by analogy with the Huggins expression:

$$k' = 14.1/(2.5)^2 = 2.25$$

Reference to Table I shows that the experimental $[\eta]$ values are of the same order as that predicted theoretically for the lower molecular weight polymers. At molecular weights of over 1500, the experimental values increase rapidly, particularly in aqueous solution. The predicted k' value falls in the middle of the experimental range. Figure 4 illustrates the way in which the experimental results fit those predicted by the equations of Einstein and Guth and Gold. In aqueous solution, polyethylene glycol 400 is the only sample which falls within the limits set by these equations, and which may, therefore, be considered as taking up a closely spherical configuration. In toluene this situation is extended as far as the polymer of molecular weight 1,000. For the samples of higher molecular

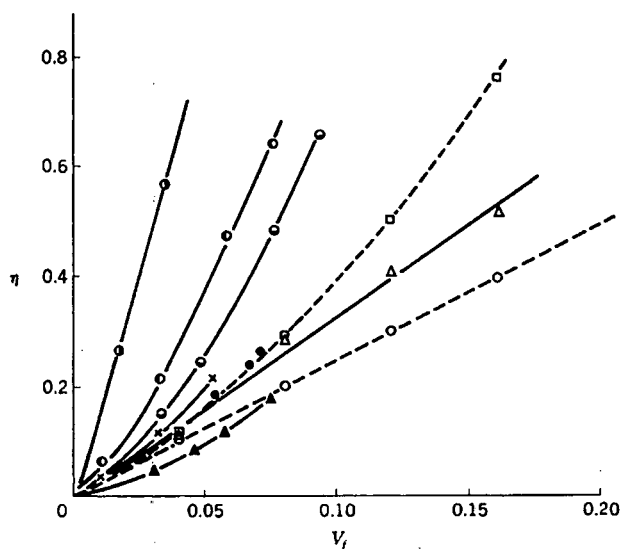


Fig. 4. Plots of η vs V_f : (○) according to the Einstein equation, $\eta_{sp} = 2.5V_f$; (□) according to the equation of Guth, Gold, and Simha, $\eta_{sp} = 2.5V_f + 14.1V_f^2$; experimental values for Carbowax 400 (Δ) in water and (▲) in toluene; Carbowax 1000 (⊙) in water and (●) in toluene; Carbowax 1500 (⊖) in water and (×) in toluene; Carbowax 4000 (⊕) in water.

weight, the assumption of spherical shape is obviously not applicable, and the molecules must be taking up a more extended configuration. From the experimental viscosities, one may evaluate the apparent volume fractions of the molecules, assuming that they were obeying the Guth and Gold relation, and compare them with the nominal values. The ratios of these volumes, evaluated for a solution in which $\eta_{sp} = 0.1$, are shown in Table II. The sequence of values obtained infers a gradual extension in the configuration of the molecule with increasing molecular weight; even so, it would appear that at a molecular weight of 4,000 the molecule is still in a highly coiled form.

TABLE II
Ratios of Nominal and Apparent Volume Fractions in Water and Toluene

Mol. Wt.	Ratio V_{app}/V_{nom} in water	Ratio V_{app}/V_{nom} in toluene
400	1.00	0.80
1000	1.40	1.00
1500	1.80	1.13
4000	4.8	3.80

A simple calculation for a molecule of molecular weight 4000 shows that the ratio of the volume in spherical form to the free volume in the fully extended form would be of the order of 500.

Huggins³ and Kuhn⁴ have proposed an expression for η_{sp} in terms of the axial ratio f and concentration for rigid, rodlike particles:

$$\eta_{sp} = (2.5 + f^2/16)V_f \tag{6}$$

This equation was deduced for systems at high dilution assuming complete Brownian movement, and would be expected to give best values for f at high dilution. Consequently, limiting values η_{sp}/V_f have been inserted in the eq. (6), and the values obtained are shown in Table III.

TABLE III
Axial Ratios Obtained for Rigid Rodlike Particles

Mol. wt	Axial ratio f (Huggins ³ and Kuhn ⁴)		Axial ratio F (Simha ⁵)	
	Water	Toluene	Water	Toluene
400	~1.0	~1.0	~1.0	~1.0
1000	3.3	~1.0	1.8	~1.0
1500	6.8	2.5	4.6	1.5
4000	12.9	7.0	10.0	4.8

From these figures it appears that the most highly extended form is that of polyethylene glycol 4000 in water with $f = 12.9$; if the molecule were fully

extended, the axial ratio should be of the order of 180. Apparently, therefore, even in this case, the molecule still takes up a highly coiled configuration, but, owing to interaction between itself and the solvent, it has departed from the roughly spherical form of the lower molecular weight polymers. The figures also bear out previous indications that in toluene the molecules are in a much less extended form.

A rather more elaborate expression has been deduced for the relation between η_{sp} , f , and V_f by Simha:⁵

$$\eta_{sp} = \left[\frac{f^2}{15 \ln[2f - 1.5]} + \frac{f^2}{5 \ln[2f - 1.5]} + \frac{14}{15} \right] V_f \quad (7)$$

Again, from limiting values of η_{sp}/V_f the axial ratio f can be calculated; these values are also shown in Table III and are essentially the same as those obtained from the simpler expression of Huggins and Kuhn.

CONCLUSIONS

Measurements of the specific viscosities of solutions of polyethylene glycols in water and toluene show that, within the molecular weight range 400–4000, a Houwink-type⁹ relationship exists between intrinsic viscosity and molecular weight. The variation of viscosity with concentration does not follow the Huggins equation in so far as the value for k' depends upon the molecular weight of the polymer. Only for polyethylene glycol with a molecular weight of 4000 in water is the expected value of k' obtained, and, so, only in this case can the requirements of the Huggins equation have been satisfied. For molecular weights of up to 1000, the correspondence between the experimental results and those predicted by the equations of Einstein and Guth and Gold, suggests that the molecules take up an approximately spherical configuration. Above this value, large departures occur which can be accounted for by assuming that the molecules exist in a more extended form. Calculations of the effective volumes of these molecules and of their axial ratios as given by the equations of Huggins, Kuhn, and Simha confirm that such extended forms do occur. The highest value obtained for the axial ratio, i.e., 12.9 for polyethylene glycol 4000 in water, must still represent quite a close configuration in view of an approximate value of 180 expected theoretically. As anticipated, the value of the axial ratio for a given polymer is always less in toluene than in water.

Thanks are due to Mr. T. A. Thomas for performing much of the experimental work.

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Synopsis

The intrinsic viscosities of a series of polyethylene glycols have been measured in water and toluene, and a relationship proposed between molecular weight and intrinsic viscosity. Values obtained for the Huggins constant below a molecular weight of 4000 are much larger than would be anticipated for linear high polymers. Some information about the variation of configuration with molecular weight has been obtained by comparing the specific viscosities with those expected from the relationships of Einstein and Guth and Gold for spherical particles, and those of Kuhn and Simha for rodlike particles.

Résumé

Les viscosités intrinsèques d'une série de polyéthylène glycols ont été mesurées, dans l'eau et le toluène, et une relation entre le poids moléculaire et la viscosité intrinsèque est proposée. Les valeurs obtenues pour la constante de Huggins à des poids moléculaires inférieurs à 4.000 sont beaucoup plus élevées qu'on aurait supposé pour des hauts polymères linéaires. On a obtenu quelques indications concernant la variation de configuration avec le poids moléculaire en comparant les viscosités spécifiques avec celles prévues sur la base des relations de Einstein, de Guth et Gold pour les particules sphériques, et celles de Kuhn et de Simha pour les particules en forme de bâtonnets.

Zusammenfassung

Die Viskositätszahlen einer Reihe von Polyäthylenglykolen sind in Wasser und Toluol gemessen worden, und ein Verhältnis zwischen Molekulargewicht und Viskositätszahl wurde vorgeschlagen. Die erhaltene Werte der Huggins-konstante sind für Molekulargewichte unter 4000 viel grösser als die für linienförmigen Hochpolymeren erwarteten. Daten über der Molekulargewichtsabhängigkeit der Konfiguration wurden durch Vergleich der spezifischen Viskositätszahlen mit den Beziehungen von Einstein und Guth und Gold vorausgesehenen Werte für sphärischen Partikeln und denen aus den Beziehungen von Kuhn und Simha für stäbchenformige Partikeln.

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Aqueous solution properties of oligo- and poly(ethylene oxide) by static light scattering and intrinsic viscosity

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Conformational properties of ethylene glycol, oligo-, and poly(ethylene oxide) ranging in weight-average molecular weight (M_w) from 62 to 1.1×10^7 have been studied by static light scattering and viscometry in salt-free and 0.45 M K_2SO_4 water and in benzene. Above $M_w = 6 \times 10^3$, intrinsic viscosity ($[\eta]$) and z -average square radius of gyration ($\langle S^2 \rangle_z$) of PEO in a salt-free solution at 25°C, $[\eta] = 4.33 \times 10^{-4} M_w^{0.67}$ dl g⁻¹ and $\langle S^2 \rangle_z = 4.08 \times 10^{-18} M_w^{1.16}$ cm², respectively. The second virial coefficient is positive in water at 25°C but one order of magnitude smaller than reported by Devanand *et al.* (*Macromolecules*, 1991, **24**, 5943). A θ -temperature for PEO in 0.45 M K_2SO_4 aqueous solution is determined to be 34.5°C and above $M_w = 4 \times 10^2$, $[\eta]$ and $\langle S^2 \rangle_z$ follow the power laws for an unperturbed polymer chain. The characteristic ratio (C_∞) of PEO is 5.2 ± 0.1 . 9.3 Å was determined for the persistence length and 8.7 Å⁻¹ for the molar mass per unit contour length of a PEO chain, based on the helical wormlike touched-bead model. The PEO chain in water behaves as a flexible polymer with a relatively large hydrodynamic diameter (9 Å) because of the hydration of the PEO chain. © 1997 Elsevier Science Ltd.

(Keywords: poly(ethylene oxide); light scattering; intrinsic viscosity)

INTRODUCTION

Poly(ethylene oxide) (PEO) is a simple and representative linear amphiphilic polymer with interesting behaviour both in solution and in the solid state¹⁻³. It dissolves not only in a large number of organic solvents but also in water. Because of the fundamental importance and wide applications^{4,5}, many authors have studied its solution properties. The studies have been carried out both in the polymer and colloid field but the behaviour of PEO in water has been less quantitatively understood, when compared with that of, say, polystyrene (PS) in organic solvents. Unusual phenomena observed in water have been exclusively ascribed to the specificity of water as a solvent.

Studies on PEO solution properties are concerned with three subjects. First, PEO exhibits in water both upper and lower critical solution temperature phenomena (UCST and LCST). Several models have been proposed, including an entropic model due to structured water, hydrogen bond formation between polymer segments and solvent, and a conformation model⁶.

The first two models are the result of specific water structuring along a PEO backbone³. There are data suggesting that two water molecules are associated with each ethylene oxide monomer unit and the diffusion coefficient of the water near a chain is substantially lower than in bulk water^{7,8}. The phase separation, however, also takes place in a non-hydrogen-bonding solvent such as *t*-butyl acetate⁹. This shows that water specificity as a solvent is not necessarily a key factor in this behaviour. Several spectroscopic studies using Raman¹⁰⁻¹² and X-ray scattering¹³, n.m.r.¹⁴⁻¹⁶, and dielectric constant measurements¹⁷ have shown that the conformation around the central OC-CO bond varies depending on the polarity of the surroundings. Recently, the local conformation of the PEO chain has caused controversy¹⁸.

The second problem concerns the tendency of PEO to aggregate in solution. The aggregation in various solvents has been reported by several research groups. For example, Polik and Burchard¹⁹ reported that PEO chains aggregate significantly in water over a wide temperature range from 20 to 90°C. Zhou and Brown²⁰ also reported that the PEO molecules aggregate in methanol at room temperature. They observed a significant downturn in the angular dependence of the light scattering, indicating the presence of some aggregates in *good solvents*. However, Kato *et al.*²¹ reported that PEO does not aggregate in water from the measurements of dynamic and static light scattering, and viscometry. Quite recently, static and dynamic light

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scattering measurements by Devanand *et al.*²² and Kinugasa *et al.*²³ found that PEO molecules do not aggregate in water, methanol, and acetonitrile at room temperature, when the solutions are meticulously prepared. The second virial coefficients reported by Devanand *et al.*²², however, are of unreasonably large values, when compared with those of PS and poly(methyl methacrylate) (PMMA) with corresponding molecular weights in good solvents.

The third subject of interest is the complexation of PEO with small cations in organic solvents. Lundberg *et al.*²⁴ reported that PEO chains behave like a polyelectrolyte in methanol when potassium iodide is added. In water, however, the complexation may not take place but the solubility power of water for PEO chains may decrease with increasing salt concentration (C_s). Here, we chose 0.45 M K_2SO_4 aqueous solution as a θ -solvent.

Over the past decade, we have been studying the polymerization behaviour of polymerizable PEO macromonomers from the viewpoint of their amphiphilic character, in order to prepare multiphase well defined graft copolymers²⁵. When one analyses the polymerization behaviour and the solution properties of the resulting graft copolymers, comblike polymers²⁶, and latex surface graft copolymers^{27,28}, an understanding of the conformational properties of a PEO chain is essential as a reference.

The present paper reports conformational properties of the PEO chain in water or benzene. We measured $[\eta]$ and $\langle S^2 \rangle_z$ of the PEO samples with a weight-average molecular weight (M_w) ranging from 62 (ethylene glycol) to 1.1×10^7 and compared it with the draft reported. The θ -temperature was determined and the data obtained under this condition were compared with the helical worklike touched-bead model (HW) developed by Yamakawa and his coworkers²⁹. The characteristic parameters of PEO chains in water including the persistence length, characteristic ratio, and hydrodynamic diameter were determined and discussed.

EXPERIMENTAL

Materials and preparation of solutions

Five samples below $M_n = 750$ were purchased from Kishida Chemical Co., Japan. The samples, PEO-2K, 4K, 5K and 20K were supplied from Nakarai Chemical Co., Japan, and PEO-10K was donated by the Takemoto Oil & Fat Co., Japan. The samples of the SE-n series were from the Tosoh Co., Japan and PEO-15H and -18H from the Seitetsu Chemical Co., Japan. The samples below $M_n = 400$ were used without further purification. The samples with molecular weights between 750 and 88×10^4 were reprecipitated three times by pouring their benzene solutions into excess hexane. The precipitated samples were filtered off, washed with hexane, and freeze-dried from benzene, and dried *in vacuo* to constant weight. PEO-18H and -15H with broad molecular weight distributions were fractionated with benzene-isooctane and purified by the above procedures. Air in the sample containers was replaced with argon and the samples kept in a freezer. All the samples were again dried *in vacuo* at least for 24 h at room temperature just before preparation of the solution. K_2SO_4 (Kishida) was dried *in vacuo* at 80°C for 24 h just before use. Water purified with a Millipore Milli Q purification system was used in all

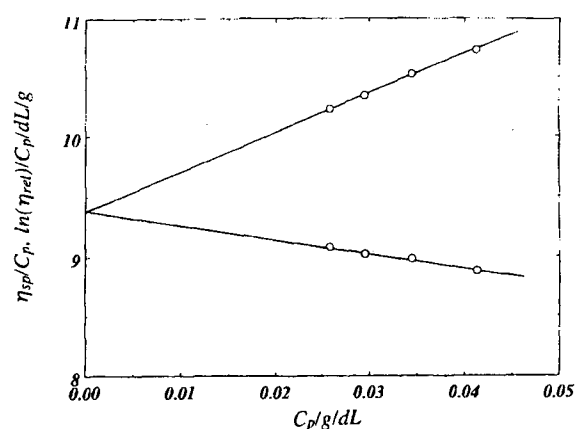


Figure 1 Plots of η_{sp}/C_p (upper) and $\ln(\eta_{rel})/C_p$ (lower) of PEO-18HD salt-free aqueous solution against polymer concentration (C_p) at 25°C

experiments. H.p.l.c. grade benzene (Kishida) for optical calibration in light scattering experiment and for viscometry was used as received. In viscometry, 0.1 vol% of allyl alcohol (Kishida) was added to the water, to avoid oxidation of PEO^{30,31}. Time-dependent viscosity measurements revealed no chain breaking even for the sample above $M_w = 10^6$.

All the solutions except for the θ -solvent were prepared by standing in the dark without shaking at room temperature at least for 3 days. All solutions were prepared gravimetrically, and their polymer mass concentrations C_p ($g\ ml^{-1}$) were calculated.

Measurements

V.p.o. Vapour pressure osmometry (v.p.o.) was run with a molecular weight apparatus from Corona Electric Co., Ltd, Type 117 at 45°C. Thiophene-free benzene was used and the instrument was calibrated with 1,2-diphenylethane (Kishida).

Viscometry. Viscometry measurements were carried out using a four-bulb low-shear capillary viscometer for molecular weights PEO higher than 10^5 and a conventional capillary viscometer of the Ubbelohde type for the lower molecular weight samples. The Huggins plot³² and the Fuoss-Mead plot³³ were combined to determine $[\eta]$. A typical example is shown in Figure 1.

G.p.c. Aqueous gel permeation chromatography (g.p.c.) was carried out at 25°C, using a HLC-8020 (Tosoh Co. Ltd), fitted with three columns (TSK-G-6000PW_{XL}, 4000PW_{XL}, and 2500PW_{XL}) and a refractive index detector. The chromatographic solvent ($1\ ml\ min^{-1}$) was water containing a small amount of sodium azide. The elution volume was calibrated with nine PEO standard samples ($150 < M_w < 2.26 \times 10^6$).

Refractive index increment. Excess refractive indices of PEO solution in the presence and absence of K_2SO_4 were measured using a differential refractometer, RM-102 from Otsuka Electronics, Co. Ltd and RF-600 from C. N. Wood Mfg. Co., Ltd. The refractive index increment (dn/dc) for a salt-free aqueous solution of PEO with $M_w = 2.24 \times 10^4$ was $0.1328\ ml\ g^{-1}$ at $\lambda = 632.8\ nm$ 25°C. The dn/dc values for undialysed PEO ($M_w = 2.24 \times 10^4$) solution containing 0.45 N K_2SO_4 were $0.119\ ml\ g^{-1}$ at 33.0°C and $0.124\ ml\ g^{-1}$ at 36.0°C.

Table 1 Results from v.p.o., g.p.c., light scattering, and viscosity measurements of ethylene glycol, oligo- and poly(ethylene oxide) samples

Code	$10^{-3}M_n$ v.p.o.	$10^{-4}M_w$ LS	M_w/M_n^b g.p.c.	$[\eta]_{H_2O}^c$ (dl g ⁻¹)	$[\eta]_{\theta}^d$ (dl g ⁻¹)	$[\eta]_{benzene}^e$ (dl g ⁻¹)	$10^{12}\langle S^2 \rangle_{z,H_2O}$ (cm ²)	$10^{12}\langle S^2 \rangle_{z,\theta}$ (cm ²)
EG	0.0621 ^a		1.00 ^a	0.022 ₁	0.021 ₄			
DEG	0.106 ^a		1.00 ^a	0.023 ₇	0.024 ₂			
TEG	0.152 ^a		1.00 ^a	0.027 ₇	0.028 ₈			
PEO-0.4K	0.40		1.0 ₆	0.040 ₀	0.034 ₀	0.028 ₃		
PEO-0.75K	0.75		1.1 ₀	0.055 ₀	0.054 ₈	0.039 ₀		
PEO-2K	1.9 ₄		1.0 ₇	0.087 ₀	0.077 ₀	0.073 ₆		
PEO-4K	3.8 ₀		1.0 ₅	0.130	0.105	0.095 ₁		
PEO-5K	5.1 ₉		1.0 ₉	0.152	0.119			
PEO-10K	12 ₂		1.0 ₉		0.181	0.219		
PEO-20K		2.2 ₄	1.1 ₅	0.395	0.253	0.376		
SE8		8.6 ₀	1.1 ₀	1.02				
SE15		15 ₀	1.0 ₈	1.52				
SE15		17 ₃	1.1 ₃	1.47	0.715	1.45 ₅	4.87	
SE30		32 ₁	1.0 ₈	2.15				
SE30		34 ₈	1.0 ₅	2.61	0.968	2.26	10.6	4.41
SE70		53 ₈	1.1 ₀	3.60	1.20	3.06	17.1	6.77
SE70		61 ₁	1.2 ₈	3.33			25.2	
SE150		86 ₈	1.1 ₇	4.74	1.52	4.93	28.1	11.1
SE150		88 ₂	1.2 ₅	4.33			32.0	
PEO-18HD		22 ₆	1.2 ₀	9.33		9.33	92.7	
PEO-15HC		44 ₆		15.1			226	
PEO-18HC		110 ₀		25.7			1450	

^a Calculated from the molecular formula
^b Calibrated with a series of PEO samples
^c At 25°C
^d In 0.45 M K₂SO₄ solution at 34.5°C
^e At 25°C

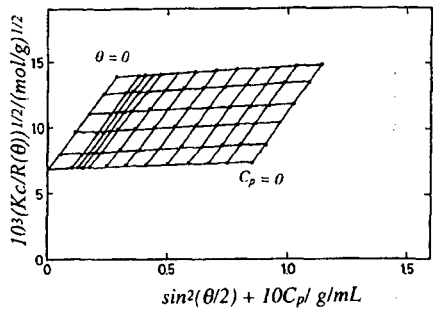


Figure 2 Berry square-root plots for PEO-20K in salt-free aqueous solution at 25°C

Light scattering Static light scattering measurements were run with a modified LS-601 (Union Gikken Co., Ltd) equipped with a vertically polarized light of a 10 mW He-Ne laser of 632.8 nm as an incident beam. For optical purification, the aqueous solution was filtered slowly and directly into light scattering cells using a microfeeder. The filters used were cellulose acetate membrane filters (0.2–1.0 μm) with a 25 mm diameter. The water used for light scattering measurement was three times filtered. The Rayleigh ratio $R(\theta)$ of the scattered light was based on the Rayleigh ratio $R_B(90) = 11.84 \times 10^{-6} \text{ cm}^{-1}$ of pure benzene at a wavelength (λ_0) of 632.8 nm³⁴. Scattered intensity data at angles (θ) were analysed by Berry square-root plots

$$\left(\frac{KC_p}{R(\theta)}\right)^{\frac{1}{2}} = \left[\frac{1}{M_w} \left(1 + \frac{16\pi^2 n_0^2}{3\lambda_0^2} \langle S^2 \rangle_z \sin^2\left(\frac{\theta}{2}\right) + 2A_2 M_w C_p + \dots \right) \right]^{\frac{1}{2}} \quad (1)$$

where n_0 is the refractive index of the solvents and A_2 , the second virial coefficient, and K is the optical constant

given as $K = 4\pi^2 n_0^2 (dn/dc)^2 / (\lambda_0^4 N_A)$ with Avogadro's number N_A .

RESULTS AND DISCUSSION

Salt-free aqueous solution

Figure 2 shows Berry square-root plots for PEO-20K in salt-free aqueous solution at 25°C. It is seen that it has a normal form without any downturn in low θ and gives a reasonable M_w value, suggesting that no aggregates exist in water at 25°C. Such results were obtained for all samples we examined by light scattering. Thus, it is fair to say that PEO does not aggregate in water at 25°C. For samples with a molecular weight below 2×10^4 , the number-average molecular weight (M_n) is determined by v.p.o. measurements and for the higher molecular weight samples M_w is determined by light scattering measurements. The molecular characteristics of the PEO samples we used are listed in Table 1. The polydispersity index (M_w/M_n) determined from g.p.c. shows that the samples, except for PEO-15HC and PEO-18HC have relatively narrow, molecular weight distributions.

The values of $[\eta]$ determined for PEO in salt-free solution at 25°C are listed in Table 1 and the double logarithmic plot (open circles) against M_w is shown in Figure 3. It can be seen that the present data are in good agreement with those (triangles) reported by Kato²¹. For $62 < M_w < 6 \times 10^3$ the slope of $\log [\eta]$ against $\log M_w$ gradually increases with increasing M_w and for $10^3 < M_w < 6 \times 10^3$ it is nearly 0.5. For $M_w > 6 \times 10^3$, the slope is almost constant giving

$$[\eta] = 4.33 \times 10^{-4} M_w^{0.67}, \quad \text{dl g}^{-1} \quad (2)$$

The values of $\langle S^2 \rangle_z$ in salt-free solution at 25°C are also summarized in Table 1 and the double logarithmic

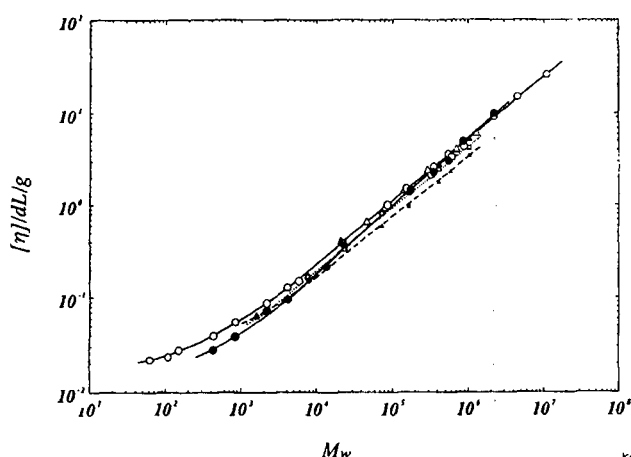


Figure 3 Molecular weight dependence of $[\eta]$ for PEO in salt-free solution at 25°C (○) and in benzene at 25°C (●). The literature data in the various solvents are shown for comparison, (Δ) in water at 25°C²¹, (▲) in benzene at 25°C³⁵, (□) in CCl₄ at 25°C³⁵, (◻) in acetone at 25°C³⁵

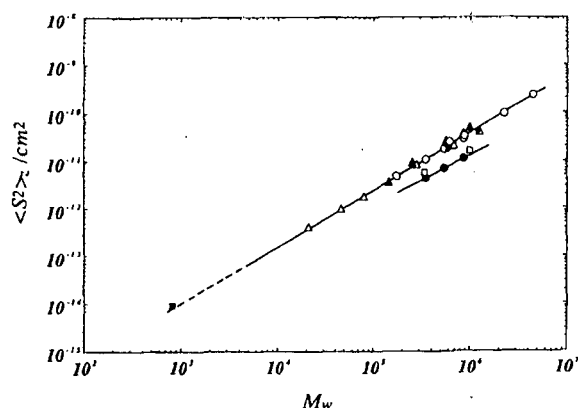


Figure 4 Molecular weight dependence of $\langle S^2 \rangle_z$ for PEO in salt-free solution at 25°C (○) and in 0.45 M K₂SO₄ solution at 34.5°C (●). The data reported are also plotted for comparison, (Δ) in water at 25°C²¹, (◻) in water at 25°C²³, (▲) in water at 30°C²², (●) in methanol at 25°C²⁰, and (■) in acetonitrile⁴¹

plot against M_w , is shown in Figure 4, together with the data reported by several groups. The present data are in good agreement with those at 30°C reported by Devanand *et al.*²² and those in MeOH at 25°C by Zhou *et al.*²⁰, but slightly higher than those in water at 25°C by Kinugasa *et al.*²³. Except for the last data, the results are fitted by

$$\langle S^2 \rangle_z = 4.08 \times 10^{-18} M_w^{1.16} \quad \text{in cm}^2 \quad (3)$$

These power laws relating $[\eta]$ and $\langle S^2 \rangle_z$ with M_w above about $M_w = 6 \times 10^3$ in water at 25°C show that a PEO chain assumes an expanded random coil conformation by an excluded-volume effect in a typical good solvent.

Comparison of PEO's $[\eta]$ in water with those in organic solvents

The $[\eta]$ values determined in benzene at 25°C are listed in Table 1 and plotted against M_w , as shown in Figure 3. The data of $[\eta]$ reported by Beech and Booth³⁵ are also plotted for comparison. There are two interesting points to be noted in this figure. First above $M_w = 1 \times 10^4$, the values of $[\eta]$ in benzene approach those in water with increasing M_w . Above $M_w = 5 \times 10^3$, the slope of a plot

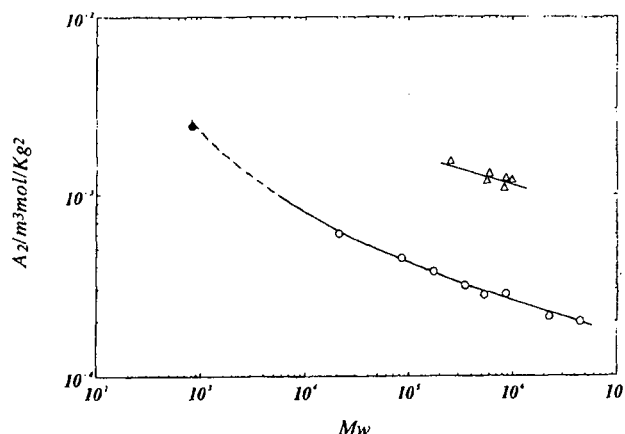


Figure 5 Molecular weight dependence of second virial coefficient (A_2) of PEO in salt-free aqueous solution at 25°C (○). The A_2 at 30°C (Δ)²² and (●) in CH₃CN⁴¹ are shown for comparison

of $\log [\eta]$ against $\log M_w$ is 0.69, in benzene, 0.66₂ in CCl₄, and 0.61₂ in acetone, respectively. Thus, benzene is the best solvent for PEO at 25°C among these. Second, for $M_w < 1 \times 10^4$, the values of $[\eta]$ in water are larger than those in the other organic solvents. This may be due either to an increase of the apparent hydrodynamic volume of PEO by hydration as will be discussed later, or to a difference in the local conformation. Below $M_w = 5 \times 10^3$, the slope in organic solvents also increases gradually with increasing M_w and to an almost constant value, just like the result observed in salt-free water at 25°C.

Second virial coefficients

Figure 5 shows a double logarithmic plot of A_2 at 25°C against M_w . The data in water at 30°C by Devanand *et al.*²² are also shown for comparison. Two points to be noted in this figure are the difference in the absolute values and in the molecular weight dependence between them. At the same M_w , the present data at 25°C which are similar to those reported for PS³⁶ and PMMA³⁷ in good solvents, are one order of magnitude smaller than those at 30°C. Devanand *et al.* interpreted the enhanced coil-coil interaction in terms of the supposedly unusual ability of water molecules to pack into and swell the coils. In fact, Bailey and his coworkers reported that the exponent of the power law in M_w -dependence of the $[\eta]$ is 0.78 at 30°C³⁸, 0.82 at 35°C³⁹, and 0.81 at 45°C³⁹, which are considerably higher than the present value (0.67₉) at 25°C. At this moment, we suspect that this big difference is due to a change in the water specificity with temperature. The studies about the influence of temperature on PEO chain conformation in water are now in progress.

The slope of A_2 against M_w in the present data in Figure 5 slowly decreases with M_w . For M_w higher than 10^3 :

$$A_2 = 3.7_2 \times 10^{-3} M_w^{-0.19 \pm 0.02} \quad \text{m}^3 \text{mol Kg}^2 \quad (4)$$

In the two-parameter theory, A_2 depends on a penetration function (Ψ), a universal function of an excluded volume parameter⁴⁰,

$$A_2 = \Psi \frac{4\pi^{3/2} N_A \langle S^2 \rangle^{3/2}}{M^2} \quad (5)$$

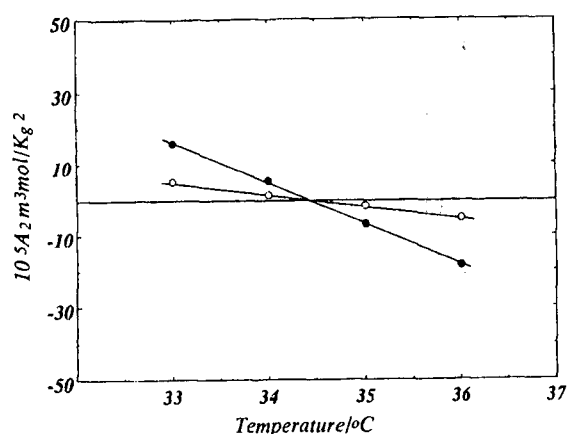


Figure 6 Temperature dependence of A_2 in 0.45 M K_2SO_4 solution of SE-70 (●) and SE-150 (○)

If Ψ asymptotically converges to a finite value at $M = \infty$, the equation is well known to predict $A_2 \sim M^{-0.2}$, in agreement with our data. Thus, the PEO chain behaves in water at 25°C like a conventional flexible linear polymer in a good solvent. We calculate Ψ using the variables determined in this work. The value for PEO-18HD is about 0.09, which is considerably smaller than the value (0.22–0.25)⁴⁰ found in PS and PMMA with a corresponding expansion factor ($\alpha^2 = \langle S^2 \rangle / \langle S^2 \rangle_\theta$).

θ -Temperature of 0.45 M K_2SO_4 solutions of PEO

To examine the solution behaviour in light of current theories, we need the θ -condition of PEO in aqueous solution. Two θ -solvent systems known for PEO are 0.45 M K_2SO_4 at 35°C³⁹ and 0.39 M $MgSO_4$ at 45°C³⁹. We adopted the former. Figure 6 shows plots of A_2 against temperature for the two PEO samples SE-70 and SE-150 in 0.45 M K_2SO_4 . It is seen that A_2 vanishes at nearly the same temperature of 34.5°C.

The $\langle S^2 \rangle_z$ and $[\eta]$ in the θ -solvent are plotted against M_w in Figures 4 and 7 (denoted by filled circles), and listed in Table 1. These plots are linear above $M_w = 400$, and by least squares we obtained

$$[\eta]_\theta = 1.57 \times 10^{-3} M_w^{0.504} \text{ dl g}^{-1} \quad (6)$$

and

$$\langle S^2 \rangle_{z\theta} = 1.11 \times 10^{-17} M_w^{1.01} \text{ cm}^2 \quad (7)$$

The characteristic ratio (C_∞) of PEO in water defined by $C_\infty = (6\langle X^2 \rangle_\theta) / (n\bar{l}^2)$ is calculated to be 5.2 ± 0.1 , where n is the number of skeletal bonds, and $\bar{l}^2$ is the average of the square of their length ($= (l_{c-c}^2 + 2l_{c-o}^2) / 3 = 2.14 \text{ \AA}^2$ with $l_{c-c} = 1.53 \text{ \AA}$ and $l_{c-o} = 1.43 \text{ \AA}$). The C_∞ value is in good agreement with that reported by several researchers^{34,41,42}, but remarkably smaller than 6.9–7.6 reported by Kugler *et al.*⁴³ and Vennemann *et al.*⁴⁴, and bigger than 4.0 reported by Flory⁴⁵.

The Flory–Fox viscosity factor (Φ_θ) for the θ -solvent is $2.4 \pm 0.1 \times 10^{23} \text{ mol}^{-1}$ from the equation

$$\Phi_\theta = \frac{[\eta]_\theta M_w}{6^{3/2} \langle S^2 \rangle_\theta^{3/2}} \quad (8)$$

which is very close to the value, $2.55 \times 10^{23} \text{ mol}^{-1}$ reported by Miyaki *et al.*⁴⁶ who investigated the PS–cyclohexane system, but slightly smaller than the recent value, $(2.79 \pm 0.08) \times 10^{23} \text{ mol}^{-1}$, reported by Konishi

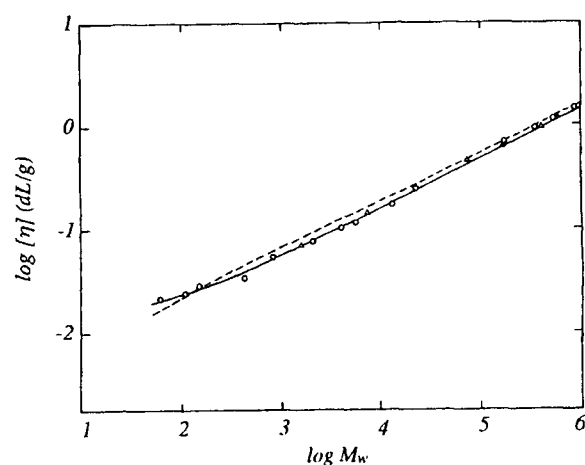


Figure 7 Comparison between the measured $[\eta]$ in 0.45 M K_2SO_4 aqueous solution at 34.5°C and helical wormlike touched-beads model. The dashed line is a theoretical curve with $\lambda^{-1}\kappa_0 = 2.4$, $\lambda^{-1} = 0.5$, $\lambda^{-1} = 12.6 \text{ \AA}$, $M_L = 7.9 \text{ \AA}^{-1}$, and $d_b = 3.2 \text{ \AA}$, and the solid line is one with $\lambda^{-1}\kappa_0 = 2.0$, $\lambda^{-1}\tau_0 = 4.0$, $\lambda^{-1} = 18.6 \text{ \AA}$, $M_L = 8.7 \text{ \AA}^{-1}$, and $d_b = 9.3 \text{ \AA}$. (○) Present work; (Δ) ref. 35

*et al.*⁴⁷. In salt-free water at 25°C, the Φ is determined as $(1.3\text{--}1.9) \times 10^{23}$ in the M_w range we examined.

Comparison with the HW model

We apply the HW model without excluded volume, developed by Yoshizaki and Yamakawa²⁹, to our intrinsic viscosity data and determine several characteristic conformation parameters of a flexible PEO chain in water. According to the theory, $[\eta]$ is written as the sum of the solution $[\eta]_{KR}$ of the Kirkwood–Riseman equation and the contribution $[\eta]_E$ of the Einstein spheres,

$$[\eta] = [\eta]_{KR} + [\eta]_E \quad (9)$$

$[\eta]_E$ and $[\eta]_{KR}$ are given by

$$[\eta]_E = 5\pi N_A N d_b^3 / 12M \quad (10)$$

$$[\eta]_{KR} = \left(\frac{\langle S^2 \rangle}{\langle S^2 \rangle_{KP}} \right)^{3/2} [\eta]_{KR}^{KP} \Gamma(L, d_b; \kappa_0, \tau_0) \quad (11)$$

where N is the number of beads, d_b is a diameter of the bead, L is the HW chain contour length, κ_0 and τ_0 are related to curvature and torsional constants for a elastic wire. $\langle S^2 \rangle_{KP}$, $[\eta]_{KR}^{KP}$, and $\langle S^2 \rangle$, are given, respectively, by

$$\langle S^2 \rangle_{KP} = \frac{6}{L} - \frac{1}{4} + \frac{1}{4L} - \frac{1}{8L^2} (1 - e^{-2L}) \quad (12)$$

$$[\eta]_{KR}^{KP} = 6^{3/2} \Phi_\infty \langle S^2 \rangle_{KP}^{3/2} / M \Gamma_{KP}(L, d_b) \quad (13)$$

$$\begin{aligned} \langle S^2 \rangle = & \frac{\tau_0^2}{v^2} \langle S^2 \rangle_{KP} + \frac{k_0^2}{v^2} \left(\frac{L}{3r} \cos \phi - \frac{1}{r^2} \cos(2\phi) \right. \\ & + \frac{2}{r^3 L} \cos(3\phi) - \frac{2}{r^4 L^2} \cos(4\phi) \\ & \left. + \frac{2}{r^4 L^2} e^{-2L} \cos(nL + 4\phi) \right) \end{aligned} \quad (14)$$

where Φ_∞ is 2.870×10^{23} . v , r , and ϕ are defined by

$$v = (\kappa_0^2 + \tau_0^2)^{1/2} \quad (15)$$

$$= (4 + v^2)^{1/2} \quad (16)$$

$$\phi = \cos^{-1}(2/r) \quad (17)$$

The numerical function forms of Γ and Γ_{KP} are given in ref. 29 and all lengths are measured in units of λ^{-1} . Double-logarithmic plots of $[\eta]$ (in dl g^{-1}) against M is given by

$$\log[\eta] = \log(M[\bar{\eta}]/\Phi_{\infty}L) - \log(\lambda^2 M_L) - 2.542 \quad (18)$$

with

$$\log M = \log L - \log(M_L/\lambda) \quad (19)$$

where $[\bar{\eta}]$ is the reduced intrinsic viscosity calculated from the equations mentioned above and M_L is the shift factor defined as the molecular weight per unit contour length of the chain. The shape of the double logarithmic plot of $M[\bar{\eta}]/\Phi_{\infty}L$ changes only with the values of κ_0 , τ_0 , and d_b . Therefore, the quantities of M_L/λ and $\lambda^2 M_L$ may be determined from a best fit of the double-logarithmic plot of the theoretical $M[\bar{\eta}]/\Phi_{\infty}L$ against L to that of the observed $[\eta]$ against M . Fujii *et al.* determined the model parameters by applying the model to the experimental data of the mean-square dipole moment $\langle \mu^2 \rangle$ of PEO in benzene at 25°C.⁴⁸ The parameters are $\lambda^{-1}\kappa_0 = 2.4$, $\lambda^{-1}\tau_0 = 0.5$, $\lambda^{-1} = 12.0 \text{ \AA}$, and $M_L = 8.8 \text{ \AA}^{-1}$. First, we use the $\lambda^{-1}\kappa_0 = 2.4$ and $\lambda^{-1}\tau_0 = 0.5$, and determine λ^{-1} , M_L , and d_b from comparison with the present viscometric results. The best fit curve (broken lines) is shown in Figure 7 and we obtain $\lambda d_b = 0.25$, $\log M_L/\lambda = 2.0$, and $\log \lambda^2 M_L = -1.3$. From these values, we determine $\lambda^{-1} = 12.6 \text{ \AA}$, $M_L = 7.9 \text{ \AA}^{-1}$, and $d_b = 3.2 \text{ \AA}$.

However, the theoretical curve does not express the experimental data quantitatively. The deviation is significant between $10^2 < M_w < 10^4$. Also, the theoretical curve cannot express the decrease in the slope of $\log[\eta]$ against $\log M_w$ with decreasing M_w . Fujii *et al.* adopted a bond dipole moment of $\mu_{CO} = 0.99\text{D}$ and a value of the characteristic ratio $C_{\infty} = 4.0$ in benzene at 40°C to obtain the set of parameters above. This μ_{CO} value is much smaller than that derived experimentally (1.07D)⁴⁹ or calculated by a MO method⁵⁰, and we obtained a larger characteristic ratio $C_{\infty} = 5.2$ in the present study. Therefore, the values of κ_0 and τ_0 in benzene may not be applicable to the present system.

Therefore, we tried to determine all the parameters from viscometry, by investigating the influence of the model parameters, $\lambda^{-1}\kappa_0$, $\lambda^{-1}\tau_0$, and λd_b on the shape of theoretical $M[\bar{\eta}]/\Phi_{\infty}L$ against L . To interpret the observed low dependence of $[\eta]$ on M_w for low M_w , the parameters set, $\lambda^{-1}\kappa_0 = 2.0 \pm 1.0$, $\lambda^{-1}\tau_0 = 4.0 \pm 1.0$, and $\lambda d_b = 0.50$ is obtained. The best fit curve (solid line) with these parameters is shown in Figure 7. The theoretical curve appears to explain well the experimental data for all M_w . We obtain $\log M_L/\lambda = 2.2$, and $\log \lambda^2 M_L = -1.6$, and determine $\lambda^{-1} = 18.6 \text{ \AA}$, $M_L = 8.7 \text{ \AA}^{-1}$, and $d_b = 9.3 \text{ \AA}$. The values of λ^{-1} and d_b determined are significantly bigger than those determined by Fujii *et al.*⁴⁸. It may be noted that a relatively large hydrodynamic diameter value (9.3 Å) is obtained. We are concerned with how the hydrodynamic volume of PEO chain changes with the solvents. If one assumes that the local conformation of PEO chain does not change from that in the θ -solvent, we can determine the values of d_b , λ^{-1} , and M_L in benzene and salt-free

water at 25°C. That is, we use a parameter set of $\lambda^{-1}\kappa_0 = 2.0 \pm 1.0$ and $\lambda^{-1}\tau_0 = 4.0 \pm 1.0$, determined in a θ -solvent, and compare with the $[\eta]$ values in the good solvents below $M_w = 8 \times 10^3$. The parameters determined are $d_b = 8.6 \text{ \AA}$, $M_L = 9.3 \text{ \AA}^{-1}$, and $\lambda^{-1} = 17 \text{ \AA}$ in salt-free water at 25°C and $d_b = 5.0 \text{ \AA}$, $M_L = 14.5 \text{ \AA}^{-1}$, and $\lambda^{-1} = 16.6 \text{ \AA}$ in benzene at 25°C. The hydrodynamic diameter in water is considerably larger than that in benzene. This may account for the fact that for $M_w < 10^4$ we observed higher $[\eta]$ in water than in benzene, as shown in Figure 3. Since the local conformation may vary with the solvent, the parameters determined in the good solvents may include errors. Nevertheless, it seems that the large hydrodynamic volume of PEO in water may be due to structuring waters along the PEO chain.

CONCLUSIONS

We report the aqueous solution properties of PEO in salt-free solution at 25°C and 0.45 M K_2SO_4 solution at 34.5°C. Static light scattering and viscometry measurements for PEO with M_w from 62 to 1.1×10^7 were carried out. The M_w -dependence of $\langle S^2 \rangle$ and $[\eta]$ reveals that the PEO assumes an expanded random coil conformation in water at 25°C when M_w is above $7 \sim 8 \times 10^3$. The second virial coefficient for the PEO in salt-free solution at 25°C is positive and comparable with that for PS and PMMA in good solvents. The molecular weight dependence of $[\eta]$ in the θ solvent was compared with HW model and the model parameters are determined $\lambda^{-1}\kappa_0 = 2.0 \pm 1.0$, $\lambda^{-1}\tau_0 = 4.0 \pm 1.0$, $\lambda^{-1} = 18.6 \text{ \AA}$, $M_L = 8.7 \text{ \AA}^{-1}$, and $d_b = 9.3 \text{ \AA}$. It is concluded that PEO in water behaves as a flexible polymer with a relatively large hydrodynamic diameter, compared with that in organic solvents.

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In Vitro and In Vivo Hematopoietic Effect of Mutant Human Granulocyte Colony-Stimulating Factor

By Masami Okabe, Makoto Asano, Tetsuro Kuga, Yoshinori Komatsu, Motoo Yamasaki, Yoshiharu Yokoo, Seiga Itoh, Makoto Morimoto, and Tetsuo Oka

About 100 derivatives of human recombinant granulocyte colony-stimulating factor (rhG-CSF) were created by various gene-mutagenic techniques, and KW-2228, in which amino acids were replaced at five positions of N-terminal region of intact rhG-CSF, was picked up and evaluated for its biologic and physicochemical properties in comparison with intact rhG-CSF. KW-2228 showed two to four times higher specific activity than that of intact rhG-CSF in mouse and/or human bone marrow progenitor cells by colony-forming unit assay in soft agar, and by cell-proliferation assay in liquid culture. KW-2228 showed a potency to increase peripheral neutrophil counts when it was administered to normal C3H/He mice by single intravenous injection. Increase of total leukocyte count and neutrophils was observed, with peak level at 8 to 12 hours at low doses (0.5 to 1.0 $\mu\text{g}/\text{mouse}$), and the highest level was maintained for 24 to 30 hours at high doses (5 to 10 $\mu\text{g}/\text{mouse}$). The granulopoietic effect of KW-2228 was examined by several doses of single course (once daily for

10 days) or multiple courses (twice daily injection for 5 days followed by cessation for 9 days on one cycle, 3 cycles in total) of treatment. KW-2228 showed higher activity than that of rhG-CSF, especially at sub-optimal doses of multiple courses of treatment. Furthermore, KW-2228 was found to be more stable physicochemically and biologically than intact rhG-CSF, especially under thermal conditions at 56°C and in the human plasma at 37°C, suggesting a protease resistancy. Pharmacokinetic study showed that plasma concentration of KW-2228 assayed for its bioactivity maintained a higher level than that of intact rhG-CSF for 60 minutes after intravenous injection of this protein to normal mice. Those results suggest that KW-2228 might show a superior in vivo hematopoietic effect to intact rhG-CSF due to its high specific activity to progenitor cells, and also due to its improved physicochemical, biologic, and pharmacokinetic stability in host animals.

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IT IS WELL-KNOWN that the proliferation and differentiation of hematopoietic progenitors are directly regulated by the colony-stimulating factors (CSFs) specific to each lineage of the progenitors.¹ In the human and mouse systems, four CSFs have been identified that stimulate the progenitors of granulocyte-macrophage lineage: granulocyte CSF (G-CSF), macrophage CSF (M-CSF), granulocyte-macrophage CSF (GM-CSF), and interleukin-3 (IL-3).^{2,3} Among these CSFs, human G-CSF was isolated and its cDNA was already cloned from tumor cell lines by two groups.^{4,5} It was shown that alternative splicing produced two different cDNAs that encode polypeptides of 177 amino acids (hG-CSFa) and 174 amino acids (hG-CSFb).⁶ The amino acid sequences of these two hG-CSF polypeptides were identical to each other, except that hG-CSFa had the insertion of three amino acids (Val-Ser-Glu) between Leu-35 and Val-36.

Recently, cDNA for rhG-CSF of 174-amino acid type was isolated from the cDNA library of lipopolysaccharide-stimulated normal human blood macrophages by our group (intact rhG-CSF).⁷ Furthermore, a number of mutations were introduced into the cDNA by in vitro mutagenesis to

analyze the relation of the structure/biologic activity.⁸ Although most of the mutants modified at the internal and C-terminal region of the molecule failed to keep rhG-CSF activity, some mutants at N-terminal region within 17 amino acids preserved or increased the activity. We picked up and evaluated KW-2228 in which Thr-1, Leu-3, Gly-4, Pro-5, and Cys-17 of hG-CSF were substituted by Ala, Thr, Tyr, Arg, and Ser, respectively, because it was found to possess a specific activity two- to threefold higher than that of intact rhG-CSF,⁸ and was also found to show an excellent granulopoietic effect in normal mice and recovery effect on leukopenia induced by chemotherapeutic agents or x-ray irradiation in mice by additional experimental studies.¹⁶ In this report, we describe in vitro colony-stimulating activity, in vitro bone marrow cell proliferation-stimulating activity, and in vivo granulopoietic activity of KW-2228 in normal mice in more detail.

MATERIALS AND METHODS

hG-CSFs. *Escherichia coli* derived intact rhG-CSF and mutein rhG-CSF KW-2228, in which N-terminal amino acids of rhG-CSF were substituted by Ala-1, Thr-3, Tyr-4, Arg-5, and Ser-17, respectively, were purified over 99% as described elsewhere,⁸ and used for all in vitro and in vivo experiments. Endotoxin contamination was less than 0.1 ng/0.1 mg of pure protein as determined by the limulus amoebocyte lysate assay.

Thermal stability. rhG-CSF, 20 $\mu\text{g}/\text{mL}$, in phosphate-buffered saline (PBS, pH 7.2) or α -minimum essential medium (α -MEM) containing 10% fetal calf serum (FCS) was incubated in a water bath at 56°C and G-CSF activity at 10, 30, 60, 120, and 240 minutes after initiation was determined by the bone marrow proliferation assay described below.

Colony-forming unit assay and bone marrow cell proliferation assay. Granulocyte colony formation by bone marrow cells of human or mouse was performed as described elsewhere⁹ by seeding 5×10^4 cells per 0.5 mL of α -MEM containing 20% FCS into 0.3% agar (Difco Laboratories, Detroit, MI) on a 24-well microplate (NUNC, no. 143982, Roskilde, Denmark). Colonies of more than 40

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cells were enumerated on day 10 for human or day 7 for mouse bone marrow cells. A unit of G-CSF activity was defined as the amount of rhG-CSF needed to produce a colony from 5×10^4 bone marrow cells. Bone marrow cell proliferation assay was performed on a 96-well microplate (NUNC, no. 167008, Roskilde, Denmark), with a modification of Ihle et al¹⁰ using nonadherent bone marrow cells instead of NFS-60 cells. rhG-CSF was serially diluted to 1:2 with 100 μ L of α -MEM containing 10% FCS and mixed with 100 μ L of bone marrow cells (5×10^5 cell/mL). The culture was incubated at 37°C for 72 hours, and 0.25 μ Ci of [³H]-thymidine (specific activity: 26 Ci/mmol, Amersham, Tokyo, Japan) was added for the last 20 hours for incorporation into the cells. The activity of each rhG-CSF was determined at the CSFs' concentration required for half maximal stimulation of this assay and compared with each other.

In vivo hematopoietic response. C3H/He mice (8 to 15 weeks old, male) were obtained from Shizuoka Agriculture Cooperative Association (Hamamatsu, Japan) and maintained under pathogen-free conditions throughout the experiments. Mice matched for age and body weight were injected with 0.2 mL of 0.5 to 10 μ g of KW-2228 intravenously (IV) or by other routes. Effects of KW-2228 were examined by several doses of single course (once daily for 10 days) or multiple course (twice daily for 5 days followed by cessation for 9 days on 1 cycle, 3 cycles in total). Peripheral leukocytes (WBC) were counted by a micro cell counter (CC-180A, Toa Medical Electronics Co, Hyogo, Japan), and differential counts were taken on smear preparations stained with Wright by MICROX auto cell-analyzer (HEG-120A, Tateishi Electronics, Kyoto, Japan).

Bioassay of plasma level of rhG-CSF. Plasma levels of rhG-CSF were assayed before and after single injection of KW-2228 (2.5 μ g) using serial plasma dilutions by 0.1 mL of medium containing 5×10^4 cells of NFS-60, which grows depending on G-CSF or multi-CSF.¹¹ The level (ng/mL) of rhG-CSF was calculated from calibration curve of each standard rhG-CSF preparation of known activity.

Biologic stability in human plasma in vitro. rhG-CSF, 20 μ g/mL, in PBS was diluted to 1 μ g/mL with fresh human plasma from healthy male adult volunteers, and incubated at 37°C for up to 50 hours with or without 10 mmol/L of protease inhibitor, p-APMSF (p-amidinophenyl methanesulfonyl fluoride hydrochloride, Wako Pure Chemicals, Osaka, Japan). Biologic activity at each timepoint was assayed by NFS-60 cells similarly to the above. The data was shown as the residual activity (percent) to initial level.

RESULTS

Granulocyte colony formation stimulated by KW-2228. First, we performed titration of KW-2228 for colony formation in 0.3% soft agar. Fifty thousand nonadherent bone marrow cells from C3H/He mice were seeded in culture containing KW-2228 preparations diluted twofold stepwise. As a control, cultures were also prepared with intact rhG-CSF of 99% purity. KW-2228 ranging from 0.039 ng/mL to 0.313 ng/mL stimulated granulocytic colony formation with good linearity as shown in Fig 1. And all of the colonies were confirmed to be granulocytic lineage by the double-esterase staining method.¹² This colony-forming activity of KW-2228 was more potent than that of intact rhG-CSF, which also stimulated granulocytic colonies at concentrations of 0.156 ng/mL to 1.25 ng/mL with good linearity. A specific activity of KW-2228 and intact rhG-CSF, one unit of which was defined as activity to stimulate the formation of one colony, was 4.0×10^8 U/mg of protein for KW-2228 and 1.2×10^8 U/mg of protein for intact rhG-CSF, respectively. KW-2228

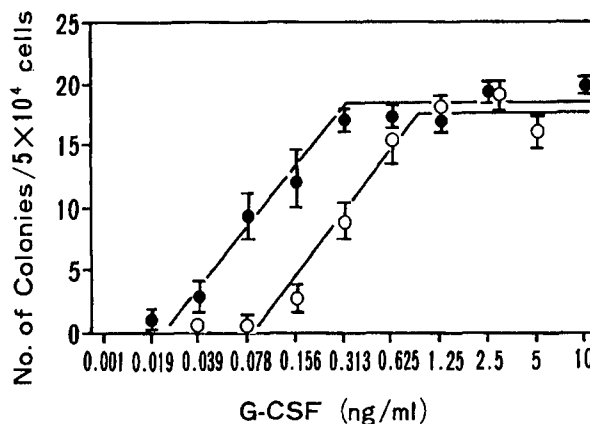


Fig 1. Granulocyte colony formation of mouse bone marrow cells by KW-2228. Twofold diluted KW-2228 (●) or rhG-CSF (○) was titrated by colony-forming unit-c (CFU-c) assay in soft agar as described in Materials and Methods. Data represent mean values and SDs (n = 4-6) of a typical experiment.

was found to be about three times more potent than that of intact rhG-CSF.

In the next experiments, femoral marrow cells were incubated with serially diluted KW-2228 sample solution in liquid microculture for 3 days. The proliferation of progenitors in femoral marrow cells determined by [³H]-thymidine uptake was stimulated by KW-2228 dose-dependently and qualitatively ranging from 0.024 ng/mL to 1.56 ng/mL (Fig 2). In this assay, KW-2228 showed about three times more potent activity than intact rhG-CSF of the same degree as obtained in the colony assay. Additionally, mature neutrophils were confirmed to be produced after 7 to 10 days' culture with KW-2228 dose-dependently by morphologic analysis of cyto-spinned preparation.

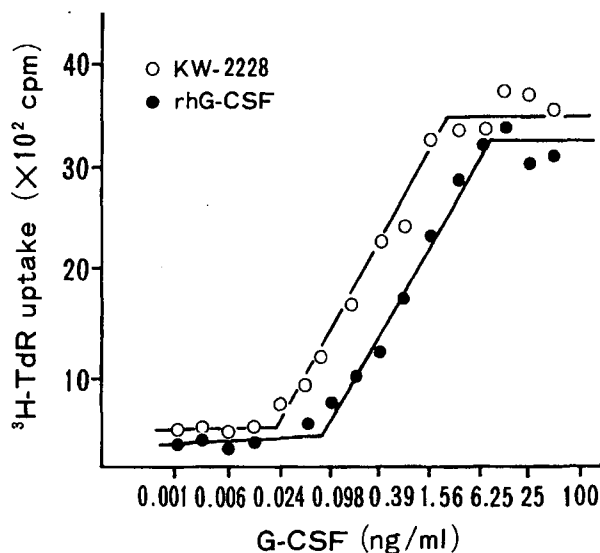


Fig 2. Stimulation of proliferation of mouse bone marrow cells by KW-2228. Twofold diluted KW-2228 (○) or rhG-CSF (●) was titrated by the proliferation assay as described in Materials and Methods. Data represent mean values (n = 3) of a typical experiment.

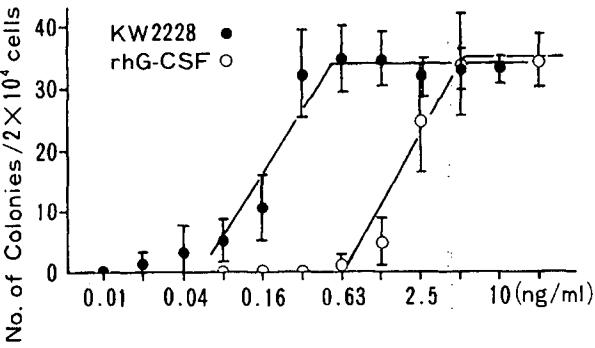


Fig 3. Granulocyte colony formation of human bone marrow cells by KW-2228. Two-fold diluted KW-2228 (●) or rhG-CSF (○) was titrated by CFU-c assay using human bone marrow cells. Data represent mean values and SDs (n = 4-6) of a typical experiment.

Effect on human femoral marrow cells was also examined by colony-forming assay or cell-proliferation assay. KW-2228 was found to show superior potency to stimulate granulocyte colony formation to intact rhG-CSF (Fig 3) and to stimulate the proliferation of human marrow cells (data not shown) in the same manner as seen in murine systems. A summary of four experiments of cell-proliferation assay, using five normal volunteers and two ovarian cancer patients who were both mildly myelo-suppressed, is shown in Table 1. KW-2228 showed about two to four times more potent activity than intact rhG-CSF against femoral marrow cells from both normal volunteers and cancer patients.

Hematopoietic effect in normal mice. C3H/He mice were given a single IV injection of 0.5 to 10 μ g of KW-2228, and peripheral neutrophils were counted at various times after the injection, as shown in Fig 4. KW-2228-treated mice developed two- to threefold rise of neutrophil counts 8 hours after administration; these levels were maintained for at least 12 hours and decreased thereafter at lower doses. On the other hand, high levels of neutrophil counts were maintained for 24 to 30 hours at higher doses. Similar pharmacokinetic effect of KW-2228 was obtained by other routes (subcutaneous, intramuscular, and intraperitoneal) of administration at a dose of 10 μ g (data not shown).

Figure 5 shows changes in differential blood cell counts by daily injections for 10 days. In comparison with single injections of KW-2228, repeated injections induced a progressive and marked rise in total leukocyte counts (Fig 5A), especially in neutrophils (Fig 5B), dose-dependently, reach-

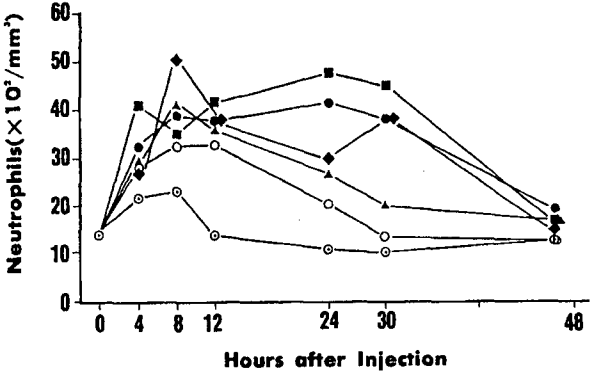


Fig 4. Granulopoietic effect of KW-2228 in normal mice. KW-2228 (10, 5, 2.5, 1.0, 0.5, 0 μ g/mouse) was injected IV into male mice (n = 4), and the number of peripheral neutrophils in each mouse was counted at indicated times after the injection.

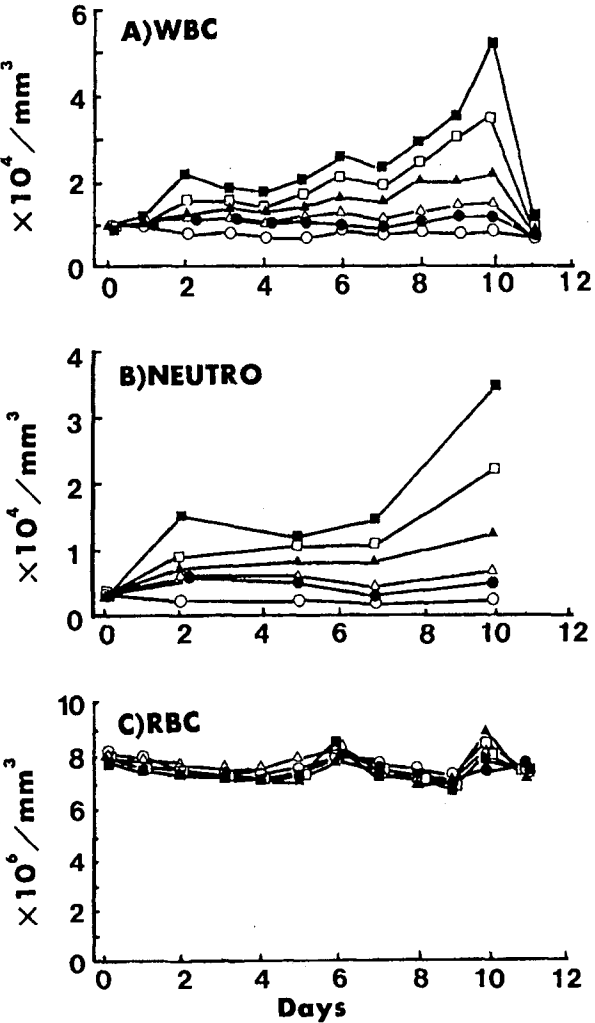


Fig 5. Effect of KW-2228 on blood cell counts in normal mice treated daily for 10 days. KW-2228 (10, 5, 2.5, 1.0, 0.5, 0 μ g/mouse) was injected IV into male mice (n = 4) daily for 10 days. Total leukocyte counts (A), neutrophil counts (B), and red blood cell counts (C) were monitored.

Table 1. Relative Activity of KW-2228 on Human Bone Marrow Cells From Healthy and Cancerous Volunteers

Drugs	CSF Activities*						
	Healthy Volunteers				Cancer Patients†		
	A	B	C	D	E	F	G
rhG-CSF	1	1	1	1	1	1	1
KW-2228	3.73	3.07	3.74	2.10	1.93	2.58	3.39

*Ratios of the proliferative activity (³H-TdR uptake) to that of intact rhG-CSF (summary of four experiments).

†Ovarian carcinoma. Relative activity of KW-2228 was calculated by comparing the half-maximum value in each dose response curve in the proliferative assay.

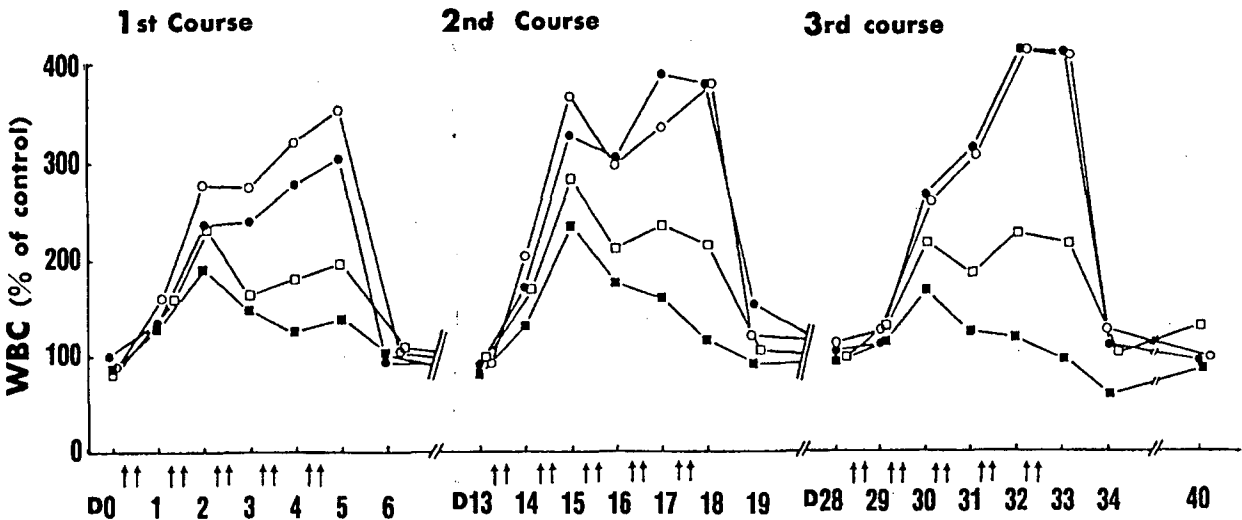


Fig 6. Total leukocyte counts in normal mice after the multiple course treatment with KW-2228. KW-2228 (○, 5; □, 1 µg/mouse) or rhG-CSF (●, 5; ■, 1 µg/mouse) was injected into male mice (n = 4) twice daily (↑↑) for 5 days (first course). The same treatment regimen was administered to the same mice starting from day 13 (second course) and from day 28 (third course), respectively.

ing a level 10 times higher than the normal one after 5 to 10 µg of consecutive doses for 10 days. Changes in red blood cell, (Fig 5C), monocyte, and lymphocyte counts (data not shown) were less marked than neutrophil counts, or somewhat constant. On the other hand, platelet counts decreased slightly and transiently at the middle phase of injection, but recovered at a later phase (data not shown). It was obvious from histologic analysis of the bone marrow and spleen that the rise in neutrophil counts of KW-2228-treated mice mainly was due to increased neutrophil granulopoiesis. However, the elevated neutrophil counts decreased to the normal level within 48 hours after the termination of injection. There was no adverse effect in main organs such as the heart, lung, kidney, or liver by histologic analysis.

Additionally, we examined the granulopoietic activity by multiple course (three cycles) of KW-2228 treatment. As shown in Fig 6, an obvious response to increasing leukocytes was obtained even in the second or third courses of treatment to the same degree as after the first course. At a lower dose of 1 µg, leukocyte-increasing effect of KW-2228 was significantly more potent than that of intact rhG-CSF, although the effect was almost equal to that at a high dose of 10 µg. Figure 7 shows the superiority of KW-2228 on leukopoietic effect in this multi-course of treatment to intact rhG-CSF of

the same dose. KW-2228 was significantly more potent than intact rhG-CSF at sub-optimal doses after both the first and the second courses, and also after the third course of treatment (data not shown).

Plasma levels of KW-2228 in mice after single injection. C3H/He mice were given a single IV injection of 2.5 µg of KW-2228 and then the plasma was collected at an interval up to 9 hours, and its bioactive content was assayed by microculture of NFS-60 cells as described in Materials and Methods. Plasma level in uninjected mice was undetectable (less than detection limit; 0.1 ng/mL). As shown in Table 2, concentration of KW-2228 in bioactive form in the plasma at 5 minutes after the injection was 143.1 ng/mL (mean of three mice) and was maintained at a high level for at least 60 minutes (177.6 ng/mL); thereafter it decreased rapidly. On the contrary, plasma level in the case of intact rhG-CSF was 75.0 ng/mL at 5 minutes, but thereafter it decreased rapidly. Thus, plasma concentration of KW-2228 in bioactive form seems to maintain a high level at early phase after IV injection and to be more stable biologically in plasma than that of intact rhG-CSF.

Thermo-stability of KW-2228. Physicochemical properties of KW-2228, such as thermo- or pH-stability, were examined and compared with intact rhG-CSF. KW-2228

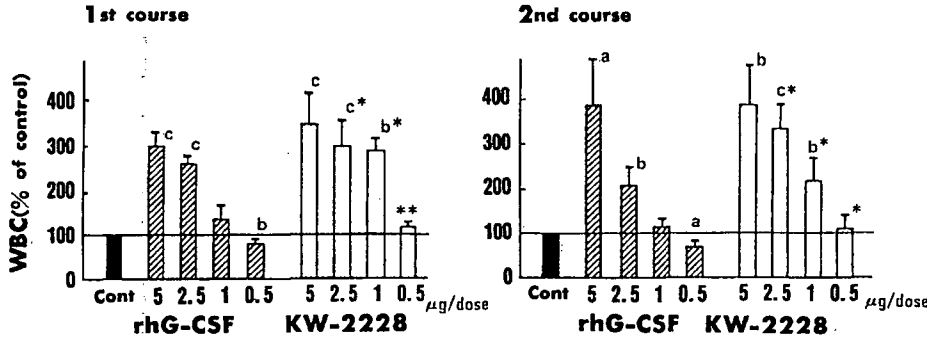


Fig 7. Total leukocyte counts in mice 5 days after the multiple course treatment with KW-2228. The treatment schedule was the same as for Fig 7. Data represent mean values and SDs (n = 5) (a, $P < .05$; b, $P < .01$; c, $P < .001$ v vehicle control; *, $P < .01$ v a respective dose of rhG-CSF).

Table 2. Plasma Level of KW-2228 in Mice After a Single Injection

Sample	n	Plasma Level (ng/mL)						
		5 min	15 min	30 min	60 min	120 min	240 min	540 min
KW-2228	3	143.1 ± 60.7	200.5 ± 55.5	221.6 ± 62.0	177.6 ± 63.1	28.4 ± 6.5	28.0 ± 4.6	13.8 ± 4.2
Intact rhG-CSF	3	75.0 ± 50.1	8.6 ± 5.4	4.3 ± 2.3	23.2 ± 14.9	12.2 ± 5.9	8.6 ± 3.0	2.7 ± 0.6

Male mice (n = 3) were injected IV with 2.5 µg of KW-2228 or rhG-CSF. Plasma was collected from each mouse at indicated times and the bioactive content was determined by bioassay using NFS-60 cells. Data represent mean values and SDs (n = 3) of a typical experiment.

was found to be more stable to the thermal condition of 56°C than intact rhG-CSF (Fig 8). A remarkable difference was observed at this treatment of these proteins, showing that about 70% of the activity of KW-2228 remained at 10 minutes, whereas an almost complete loss of activity was observed in the case of intact rhG-CSF. This thermostability was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (M. Yamazaki, unpublished data, October 1988).

Biologic stability of KW-2228 in human plasma in vitro. In the human plasma at 37°C, KW-2228 was found to be entirely stable for up to 50 hours, whereas rhG-CSF was unstable, showing gradual reduction of its activity (as seen in Fig 9). Furthermore, this reduction of activity was completely blocked by the treatment with protease inhibitor, p-APMSF, suggesting that a kind of protease in the plasma might contribute to the digestion of rhG-CSF and that KW-2228 might be resistant to such proteases.

DISCUSSION

In this study, we evaluated the biologic and physicochemical properties of KW-2228, in which amino acids were replaced at five positions of the N-terminal region of human G-CSF by gene-mutagenic techniques.⁸

KW-2228 showed two to four times higher specific activity to mouse bone marrow progenitor cells by colony-forming unit assay in soft agar (Fig 1) and by cell-proliferation assay in liquid culture (Fig 2) than that of intact rhG-CSF.⁷ This high specific activity of KW-2228 was also confirmed in the

case using human bone marrow progenitor cells from normal and ovarian cancer patients (Fig 3, and Table 1). Thus, KW-2228 showed a superior potency to intact rhG-CSF at in vitro system without any species specificity between human and mouse systems, as shown previously.¹³ Furthermore, sugar moiety¹⁴ of human G-CSF did not seem to be necessary to its biologic activity because there was no significant difference between natural and bacterial human G-CSFs (data not shown).

Peripheral neutrophil counts showed a more than 10-fold increase, with a concomitant sixfold increase in total leukocytes, at maximal level by a daily injection of KW-2228 (10 µg/mouse) for 10 days (Fig 5). No significant changes in erythrocyte, lymphocyte, or monocyte counts were observed during the course of continuous KW-2228 treatment, although a transient thrombocytopenia at high-dose was noted (data not shown). Neutrophil counts increased 2.5 times within 12 hours after a single IV injection of KW-2228 (Fig 4), and this high level was maintained for 24 to 30 hours at higher doses (5 to 10 µg/mouse).

This granulopoietic effect of KW-2228 might not be due to contamination by endotoxin, because mock G-CSF containing about 100-fold endotoxin equivalent to the highest dose of KW-2228 did not induce neutrophilia in mice in another experiment (data not shown). The response to KW-2228 might be too early to produce neutrophils if the progenitor cells in femoral marrow responded to hG-CSF with only proliferation and differentiation, like in vitro systems. The

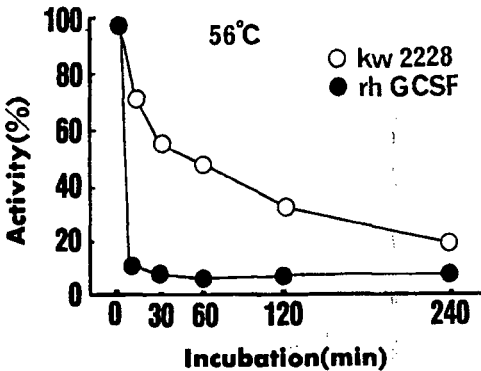


Fig 8. Thermo-stability of KW-2228. KW-2228, 20 µg/mL, (○) or rhG-CSF (●) in medium was incubated at 56°C for 240 minutes. The remaining bioactivity (% of initial) of each G-CSF was determined by the proliferation assay using mouse bone marrow cells at indicated times. Data represent mean values (n = 3) at each timepoint of a typical experiment.

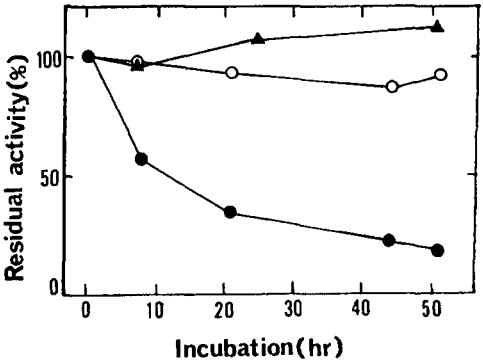


Fig 9. Stability of KW-2228 in the human plasma with or without protease inhibitor, p-APMSF. G-CSF, 20 µg/mL, was diluted to 1 µg/mL with human fresh plasma, and incubated at 37°C for 50 hours with ([○], rhG-CSF) or without ([▲], KW-2228; [●], rhG-CSF) 10 mmol/L p-APMSF. Residual bioactivity (percent of initial level) was determined by G-CSF-dependent NFS-60 cells. Data represent mean values (n = 3) at each timepoint, and SDs did not exceed 15%.

data suggest that KW-2228 might stimulate mobilization of preformed mature neutrophils from the bone marrow cavity or other pooling organs such as blood vessels. This marked elevation of neutrophil counts at the later period of injection might reflect real rhG-CSF activity, as shown in *in vitro* proliferation and colony-formation assay.

This granulopoietic effect seemed to be associated with an increase in the total number of colony-forming progenitor cells in the spleen and bone marrow. Furthermore, KW-2228 showed a superior potency to intact rhG-CSF, with significance at sub-optimal doses of a multiple course of treatment as shown in Figs 6 and 7.

The plasma level of KW-2228, at least in bioactive form, in mice injected with KW-2228 at a sub-optimal dose of 2.5 μ g/mouse, was found to be maintained higher for 60 minutes than that of intact rhG-CSF (Table 2). This difference in the plasma level might contribute to a superior *in vivo* granulopoietic effect of KW-2228 to intact rhG-CSF.

In fact, this pharmacokinetic difference was supported by the other experimental results of the stability test in human plasma (Fig 9), as well as that under thermal condition at 56°C (Fig 8). Superior stability of KW-2228 in the plasma was also confirmed in the case of the mouse (data not shown). These superior properties of KW-2228 may contribute to the increased activity *in vivo* and *in vitro*.

Concerning the receptor of KW-2228, a recent experimen-

tal result reported that KW-2228 showed the higher specific binding to the receptor on human neutrophils.¹⁵ Substitution of amino acids in the N-terminal sequence of rhG-CSF like KW-2228 might contribute to its physicochemical and biologic properties, and also in part to its high specific activity, and these in turn might contribute to *in vivo* granulopoietic activity as shown in this study.

KW-2228 was found to have the more excellent activity to recover from leukopenias induced by several chemotherapeutic agents, such as mitomycin C or cyclophosphamide, and/or induced by x-ray irradiation in mice other than the derivatives of hG-CSF.¹⁶ There was no adverse effect in mice and cynomolgus monkeys by repeated injections of KW-2228.¹⁷

Although the true mechanism for the high specific activity and physicochemical properties of KW-2228 remains to be elucidated, this is the first example of mutant rhG-CSF with high specific activity, high stability, and high affinity to its receptor, and the results in this study suggest that KW-2228 will be feasible to clinical trials.

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Add the following:

Nartograstim (Genetical Recombination)

ナルトグラスチム(遺伝子組換え)

MAPTyrASSL PQSFLKLSLE QVRKIQGDGA ALQEKLCATY KLCHPEELVL
LGHSLGIPWA PLSSCPQSAL QLAGCLSQLH SGLFLYQGLL QALEGISPEL
GPTLDTLQLD VADFATTIWQ QMEELGMAPA LQPTQGAMPA FASAFQRRAG
GVIVASHLQS FLEVSyrVLR HLAQP

C₈₅₀H₁₃₄₄N₂₂₆O₂₄₅S₈: 18905.65
[134088-74-7]

Nartograstim (Genetical Recombination) is an aqueous solution in which a desired product is a recombinant human granulocyte colony-stimulating factor (G-CSF) analog. It is N-methionylated, and threonine, leucine, glycine, proline and cysteine residues at the positions 1, 3, 4, 5 and 17 of G-CSF are substituted by alanine, threonine, tyrosine, arginine and serine, respectively. It is a glycoprotein consisting of 175 amino acid residues. It has a stimulating effect on neutrophil production.

It contains not less than 0.9 mg and not more than 2.1 mg of protein per mL, and not less than 4.0 × 10⁸ units per mg of protein.

Description Nartograstim (Genetical Recombination) occurs as a clear and colorless liquid.

Identification (1) To a suitable amount of Nartograstim (Genetical Recombination) add tris-sodium chloride buffer solution, pH 8.0 so that each mL contains 1.μg of protein, and use this solution as the sample solution. Put 0.1 mL of the sample solution in the well of a microplate for antigen-antibody reaction test, allow to stand at 5°C for not less than 10 hours, then remove the liquid, and wash the well. Then to the well add 0.25 mL of blocking TS for nartograstim test, and allow to stand at room temperature for 1 hour. Remove the blocking TS, add 0.1 mL of rabbit anti-nartograstim antibody TS to the well, and stir gently at room temperature for 3 hours. Remove the rabbit antinartograstim antibody TS, and wash the well. Then, add 0.1 mL of peroxidase labeled anti-rabbit antibody TS, stir gently at room temperature for 2 hours, remove the TS, and wash the well. Then, add 0.1 mL of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt TS, allow to stand at room temperature for 10 minutes, add 0.1 mL of a solution of oxalic acid dihydrate (1 in 50), and name this well as the sample well. Separately, proceed with 0.1 mL of tris-sodium chloride buffer solution, pH 8.0 in the same manner as for the sample solution, and name the well so obtained as the control well. When compare the sample well and the control well, the sample well reveals a green color, while the control well reveals no color.

Washing procedure of well: To the well add 0.25 mL of washing fluid for nartograstim test, allow to stand for 3

minutes, and remove the washing fluid. Repeat this procedure 2 times more.

(2) To a suitable amount of Nartograstim (Genetical Recombination) add water so that each mL contains 1 mg of protein. Replace the solvent of 2 mL of this solution with tris-calcium chloride buffer solution, pH 6.5. To 0.5 mL of the solution so obtained add 0.5 mL of tris-calcium chloride buffer solution, pH 6.5 and 5 μL of thermolysin solution (1 in 1000), allow to stand at 37°C for 21 hours, and use this solution as the sample solution. Separately, proceed with 2 mL of Nartograstim RS in the same manner as for the sample solution, and use the solution so obtained as the standard solution. Perform the test with 20 μL each of the sample solution and standard solution as directed under Liquid Chromatography <2.01> according to the following conditions, and compare these chromatograms: the similar peaks appear at the same retention times.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 220 nm).

Column: A stainless steel column 6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 35°C.

Mobile phase A: A mixture of water and trifluoroacetic acid (1000:1).

Mobile phase B: A mixture of acetonitrile, water and trifluoroacetic acid (900:100:1).

Flowing of the mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

Time after injection of sample (min)	Mobile phase A (vol%)	Mobile phase B (vol%)
0 – 5	100	0
5 – 90	100 → 40	0 → 60

Flow rate: 1.0 mL per minute.

System suitability—

System performance: When the procedure is run with 20 μL of the standard solution under the above conditions, the number of the peak which shows not less than 1.6 of the resolution between the adjacent peaks is not less than 15.

pH <2.54> 7.0 – 7.5

Purity (1) Related substances—To a suitable amount of Nartograstim (Genetical Recombination) add the buffer solution for nartograstim sample so that each mL contains about 0.5 mg of protein, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add the buffer solution for nartograstim sample to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and standard solution by SDS polyacrylamide gel electrophoresis, using buffer solution for SDS polyacrylamide gel electrophoresis and polyacrylamide gel for nartograstim. After elec-

trophoresis, immerse the gel in a solution of coomassie brilliant blue R-250 in a mixture of water, ethanol (95) and acetic acid (100) (5:4:1) (1 in 1000), and stir gently at room temperature for not less than 12 hours. Then, remove the color from the gel in a mixture of water, ethanol (95) and acetic acid (100) (13:5:2), and dry the gel under reduced pressure. Determine the areas of the colored bands obtained from the sample solution and standard solution by a densitometer at the measure wavelength 560 nm and the control wavelength 400 nm: the total area of the band other than the principal band obtained from the sample solution is not larger than the band area obtained from the standard solution.

(2) Host-derived protein Being specified separately.

(3) DNA Being specified separately.

Bacterial endotoxins <4.01> Less than 0.62 EU/ μ g.

Molecular mass To a suitable amount of Nartograstim (Genetical Recombination) add reduction buffer solution for nartograstim sample so that each mL contains about 0.5 mg of protein, and use this solution as the sample solution. Separately, to 50 μ L of molecular mass marker for nartograstim test add reduction buffer solution for nartograstim sample to make 1.0 mL, and use this solution as the standard solution. Perform the test with 10 μ L each of the sample solution and standard solution, both previously warmed at 40°C for 15 minutes, by SDS polyacrylamide gel electrophoresis, using buffer solution for SDS polyacrylamide gel electrophoresis and polyacrylamide gel for nartograstim. After electrophoresis, immerse the gel in a solution of coomassie brilliant blue R-250 in a mixture of water, ethanol (95) and acetic acid (100) (5:4:1) (1 in 1000), and stir gently at room temperature for not less than 12 hours. Then, destain the gel with a mixture of water, ethanol (95) and acetic acid (100) (13:5:2), and dry the gel under reduced pressure. Prepare a calibration curve from the migration distance of the molecular mass markers of the standard solution by plotting the migration distance on the horizontal axis and logarithm of the molecular mass on the vertical axis. Calculate the molecular mass of the sample solution from the calibration curve: the molecular mass of the main band is between 17,000 and 19,000.

Compositions ratio of related substance Being specified separately.

Assay (1) Protein content—To exactly V_1 mL of Nartograstim (Genetical Recombination) add exactly V_2 mL of water so that each mL contains about 0.5 mg of protein, and centrifuge. Determine the absorbance, A , of the supernatant liquid at the absorption maximum at about 280 nm as directed under Ultraviolet-visible Spectrophotometry <2.24>.

Amount (mg) of protein in 1 mL of Nartograstim (Genetical Recombination)

$$= A/8.71 \times (V_1 + V_2)/V_1 \times 10$$

8.71: Specific absorbance

(2) Specific activity—To a suitable exact amount of Nartograstim (Genetical Recombination) add potency measur-

ing medium for nartograstim test so that the potency is equivalent to 50% to 150% of the relative potency of the standard solution according to the expected potency, and use this solution as the sample solution. Separately, to a suitable exact amount of Nartograstim RS add an exact amount of the potency measuring medium for nartograstim test so that each mL contains exactly 1.2×10^4 units of nartograstim, and use this solution as the standard solution. Culture NFS-60 cells with subculture medium for nartograstim test, centrifuge the medium, remove the supernatant liquid by suction, and wash the cells with the potency measuring medium for nartograstim test. Repeat the washing procedure twice more, prepare two cell suspensions, containing 8×10^5 cells per mL and 4×10^5 cells per mL in the potency measuring medium for nartograstim test, and use these solutions as the cell suspension (1) and (2), respectively. In 8 wells of the 12th column of a 8×12 well-microplate put 50 μ L each of the cell suspension (1), and in all wells of the 1st to 11th columns put 50 μ L each of the cell suspension (2). Where, the wells of the 1st and 8th lines are not used for the test. To the wells of the 2nd to 4th lines of the 12th column add 50 μ L each of the standard solution, and to the wells of 5th to 7th lines of the 12th column add 50 μ L each of the sample solution. From the wells of the 12th column take 50 μ L each of the content liquid and transfer to the corresponding wells of the 1st column. Then, from the wells of the 1st column take 50 μ L each of the content liquid and transfer to the corresponding wells of the 2nd column. Proceed in the same way sequentially to the 10th column to prepare two-fold serial dilution wells. The wells of the 11th column are not performed any process. Incubate the plate under the atmosphere of 5 vol% carbon dioxide at 37°C for about 40 hours. After incubation, add to the all wells 10 μ L each of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide TS, and allow to stand under the atmosphere of 5 vol% carbon dioxide at 37°C for 4–6 hours. Add 0.125 mL of dimethylsulfoxide, shake for 5 to 10 minutes, then determine the absorbances of all wells at 550 nm and 660 nm, A_1 and A_2 , using a spectrophotometer for microplate, and calculate the difference, ($A_1 - A_2$). Divide by 6 the total of the differences ($A_1 - A_2$) of six wells of the 11th and the 1st column, which were added the standard solution, and use the value so obtained as the 50% absorbance, A_M . Determine the dilution index numbers (column number) of the two serial wells of the sample solution and standard solution, they are corresponding to just the before and after of the 50% absorbance (A_M), n_{T1} , n_{T2} and n_{S1} , n_{S2} , respectively, where $n_{T1} < n_{T2}$ and $n_{S1} < n_{S2}$. Differences of the absorbance of the serial wells are named as A_{T1} , A_{T2} and A_{S1} , A_{S2} , respectively. Calculate the relative potencies of each sample solution by the following equation using the mean value of the three standard solutions, and average them. Perform the same procedure by reversing the place of the sample solution and the standard solution. Then, calculate the mean relative potency by averaging both values.

Relative potency of the sample solution = $\frac{2^a}{\Sigma 2^b \times \frac{1}{3}}$

- a: $n_{T1} + (A_{T1} - A_M)/(A_{T1} - A_{T2})$
- b: $n_{S1} + (A_{S1} - A_M)/(A_{S1} - A_{S2})$

Obtain the potency per mL by the following equation, and calculate the potency per mg of protein using the protein content obtained in (1).

Amount (unit) of nartograstim per mL of Nartograstim (Genetical Recombination)
= $S \times$ mean relative potency of the sample solution $\times d$
S: Concentration (unit/mL) of the standard solution
d: Dilution factor for the sample solution

System suitability—

The absorbance difference of the individual wells of the standard solution of the 3rd column should be not less than A_M , and that of the individual wells of the 8th column should be not more than A_M . If they do not meet the requirements, prepare the standard solution of the range of 1.0×10^3 to 1.6×10^4 units, and perform the test.

Containers and storage Containers—Tight containers.
Storage—Light-resistant, and at not exceeding -20°C .

Add the following:

Nartograstim for Injection (Genetical Recombination)

注射用ナルトグラスチム(遺伝子組換え)

Nartograstim for Injection (Genetical Recombination) is a preparation for injection which is dissolved before use.

It contains not less than 90.0% and not more than 110.0% of the labeled amount of nartograstim (genetical recombination) ($\text{C}_{850}\text{H}_{1344}\text{N}_{226}\text{O}_{245}\text{S}_8$; 18905.65).

Method of preparation Prepare as directed under Injections, with Nartograstim (Genetical Recombination).

Description Nartograstim for Injection (Genetical Recombination) occurs as white, masses or powder.

Identification Dissolve the content of 1 container of Nartograstim for Injection (Genetical Recombination) in 1 mL of tris-sodium chloride buffer solution, pH 8.0. To a suitable amount of this solution add tris-sodium chloride buffer solution, pH 8.0 so that each mL contains 1 μg of Nartograstim (Genetical Recombination), and use this solution as the sample solution. Then, proceed with the sample solution as directed in the Identification (1) under Nartograstim (Genetical Recombination).

pH <2.54> The pH of a solution of Nartograstim for Injection (Genetical Recombination) in water, containing 100 μg

of Nartograstim (Genetical Recombination) in each mL, is 4.0 to 5.5.

Purity (1) Clarity and color of solution—A solution of Nartograstim for Injection (Genetical Recombination) in water, containing 100 μg of Nartograstim (Genetical Recombination) in each mL, is clear and colorless.

(2) Lactose conjugate—Being specified separately.

Water <2.48> Not more than 3.0% (50 mg, coulometric titration).

Bacterial endotoxins <4.01> Less than 0.62 EU/ μg .

Uniformity of dosage unit <6.02> It meets the requirement of the Mass variation test.

Foreign insoluble matter <6.06> Perform the test according to Method 2, using 3 mL of water for injection per 1 Nartograstim for Injection (Genetical Recombination) to dissolve the content: it meets the requirement.

Insoluble particulate matter <6.07> It meets the requirement.

Sterility <4.06> Perform the test according to the Membrane filtration method, using the sample solution prepared by dissolving the sample in water in a concentration to be used for the injection: it meets the requirement.

Specific activity Nartograstim for Injection (Genetical Recombination), when perform the assay and the following test, contains not less than 4.0×10^8 units of nartograstim (genetical recombination) per mg nartograstim (genetical recombination).

Wash out each content of 10 Nartograstim for Injection (Genetical Recombination) with a suitable amount of potency measuring medium for nartograstim test, wash the empty containers with the same medium, combine all washings, and add the same medium to make exactly 50 mL. To an exact amount of this solution add the same medium so that the concentration of nartograstim (genetical recombination) is equivalent to 50% to 150% of that of the standard solution, and use this solution as the sample solution. Separately, weigh accurately a suitable amount of Nartograstim RS, dissolve in the potency measuring medium for nartograstim test so that each mL contains exactly 1.2×10^4 units of nartograstim according to the labeled unit, and use this solution as the standard solution. Then, determine the nartograstim potency (unit) in 1 Nartograstim for Injection (Genetical Recombination) by proceeding as directed in the Assay (2) under Nartograstim (Genetical Recombination), and calculate the ratio against the amount of nartograstim obtained in the Assay.

Nartograstim (genetical recombination) potency (unit) in 1 Nartograstim for Injection (Genetical Recombination)
= $S \times$ mean relative potency of the sample solution $\times d \times 5$

S: Concentration (unit/mL) of the standard solution
d: Dilution factor for the sample solution

5: Amount (mL) of the medium used to dissolve per 1

sample

$$\text{Relative activity of sample solution} = \frac{2^a}{\Sigma 2^b \times \frac{1}{3}}$$

$$a: n_{T1} + (A_{T1} - A_M)/(A_{T1} - A_{T2})$$

$$b: n_{S1} + (A_{S1} - A_M)/(A_{S1} - A_{S2})$$

System suitability—

Proceed as directed in the system suitability in the Assay (2) under Nartograstim (Genetical Recombination).

Assay Weigh accurately the mass of each content of not less than 10 Nartograstim for Injection (Genetical Recombination). Weigh accurately an amount of the content, equivalent to about 0.25 mg of Nartograstim (Genetical Recombination) according to the labeled amount, dissolve in exactly 5 mL of the mobile phase, and use this solution as the sample solution. Separately, dissolve a suitable amount of Nartograstim RS in the mobile phase so that each mL contains about 50 µg of nartograstim, and use this solution as the standard solution. Perform the test with exactly 100 µL each of the sample solution and standard solution as directed under Liquid Chromatography <2.01> according to the following conditions, and determine the peak areas, A_T and A_S , of nartograstim of these solutions.

Amount (µg) of nartograstim (genetical recombination) in 1 Nartograstim for Injection (Genetical Recombination)

$$= M_S \times A_T/A_S \times M/M_T \times 5$$

M_S : Amount (µg) of nartograstim in 1 mL of the standard solution

M : Mean mass (mg) of each content

M_T : Amount (mg) of Nartograstim for Injection (Genetical Recombination)

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 280 nm).

Column: A stainless steel column 7.8 mm in inside diameter and 30 cm in length, packed with porous silica gel for liquid chromatography (5 µm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: Dissolve 15.6 g of sodium dihydrogen phosphate dihydrate and 1.0 g of sodium lauryl sulfate in 700 mL of water, adjust to pH 6.5 with sodium hydroxide TS, and add water to make 1000 mL.

Flow rate: Adjust the flow rate so that the retention time of nartograstim is about 16 minutes.

System suitability—

System performance: When the procedure is run with 100 µL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of nartograstim are not less than 3000 and not more than 2.0, respectively.

System repeatability: When the test is repeated 6 times with 100 µL of the standard solution under the above operating conditions, the relative standard deviation of the peak

area of nartograstim is not more than 1.5%.

Containers and storage Containers—Hermetic containers.

Storage—Light-resistant, and at a temperature not exceeding 10°C.

Nateglinide

ナテグリニド

Change the Description as follows:

Description Nateglinide occurs as a white crystalline powder.

It is freely soluble in methanol and in ethanol (99.5), sparingly soluble in acetonitrile, and practically insoluble in water.

It dissolves in dilute sodium hydroxide TS.

It shows crystal polymorphism.

Add the following:

Nifedipine Extended-release Capsules

ニフェジピン徐放カプセル

Nifedipine Extended-release Capsules contain not less than 93.0% and not more than 107.0% of the labeled amount of nifedipine ($C_{17}H_{18}N_2O_6$; 346.33).

Method of preparation Prepare as directed under Capsules, with Nifedipine.

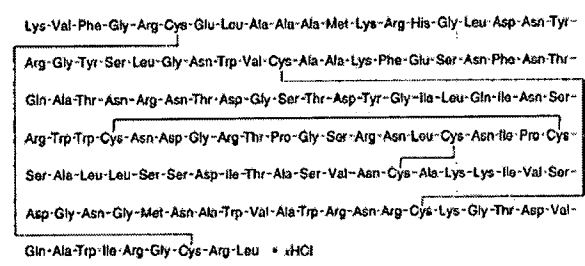
Identification Conduct this procedure without exposure to light, using light-resistant vessels. Take out the content of Nifedipine Extended-release Capsules, and powder. To an amount of the powder, equivalent to 3 mg of Nifedipine, add 100 mL of methanol, shake for 15 minutes, and centrifuge. Determine the absorption spectrum of the supernatant liquid so obtained as directed under Ultraviolet-visible Spectrophotometry <2.24>: it exhibits a broad absorption maximum between 335 nm and 356 nm.

Uniformity of dosage units <6.02> Perform the test according to the following method: it meets the requirement of the Content uniformity test.

Conduct this procedure without exposure to light, using light-resistant vessels. To the total content of 1 capsule of Nifedipine Extended-release Capsules, add 50 mL of a mixture of methanol and water (9:1), agitate for 15 minutes with the aid of ultrasonic waves with occasional shaking, and shake for further 15 minutes. Then, add methanol to make exactly V mL so that each mL contains about 0.1 mg of nifedipine ($C_{17}H_{18}N_2O_6$). Filter this solution through a membrane filter with a pore size not exceeding 0.45 µm. Discard the first 10 mL of the filtrate, pipet 5 mL of the subsequent filtrate, add methanol to make exactly 10 mL, and use this

Lysozyme Hydrochloride

リゾチーム塩酸塩



C₆₁₆H₉₆₃N₁₉₃O₁₈₂S₁₀•xHCl
[12650-88-3, egg white lysozyme]

Lysozyme Hydrochloride is a hydrochloride of a basic polypeptide obtained from albumen of hen's egg, and has an activity to hydrolyze mucopolysaccharides. It contains not less than 0.9 mg (potency) of lysozyme per mg, calculated on the dried basis.

Description Lysozyme Hydrochloride occurs as white, crystals, or crystalline or amorphous powder. It is freely soluble in water, and practically insoluble in ethanol (99.5). It is hygroscopic. The pH of a solution of Lysozyme Hydrochloride (3 in 200) is between 3.0 and 5.0.

Identification (1) To 5 mL of a solution of Lysozyme Hydrochloride in acetate buffer solution, pH 5.4 (1 in 500) add 1 mL of ninhydrin TS, and heat for 10 minutes: a blue-purple color develops. (2) Determine the absorption spectrum of a solution of Lysozyme Hydrochloride in acetate buffer solution, pH 5.4 (1 in 10,000) as directed under Ultraviolet-visible Spectrophotometry <2.24>, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

Purity (1) Clarity of solution—To 5 mL of a solution of Lysozyme Hydrochloride (3 in 200) add, if necessary, dilute hydrochloric acid to adjust the pH to 3: the solution is clear. (2) Heavy metals <1.07>—Proceed with 1.0 g of Lysozyme Hydrochloride according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 20 ppm).

Loss on drying <2.41> Not more than 8.0% (0.1 g, 105°C, 2 hours).

Residue on ignition <2.44> Not more than 2.0% (0.5 g).

Nitrogen Perform the test as directed under Nitrogen Determination <1.08>: the amount of nitrogen (N: 14.01) is between 16.8% and 18.6%, calculated on the dried basis.

Assay Weigh accurately an amount of Lysozyme Hydrochloride, equivalent to about 25 mg (potency), dissolve in phosphate buffer solution, pH 6.2 to make exactly 100 mL. Pipet 2 mL of this solution, add phosphate buffer solution, pH 6.2 to make exactly 50 mL, and use this solution as the sample solution. Separately, weigh accurately an amount of

Lysozyme Reference Standard (separately determine its loss on drying <2.41> in the same manner as Lysozyme Hydrochloride), equivalent to about 25 mg (potency), and dissolve in phosphate buffer solution, pH 6.2 to make exactly 100 mL. Pipet 1 mL and 2 mL of this solution, add phosphate buffer solution, pH 6.2 to them to make exactly 50 mL, and use these solutions as the standard solution (1) and the solution (2), respectively. Keep the sample solution and the standard solutions in an ice-bath. Pipet 4 mL of substrate solution for lysozyme hydrochloride, previously warmed in a water bath of 35°C for about 5 minutes, add exactly 100 μL of the sample solution, previously warmed in a water bath of 35°C for about 3 minutes, and allow to stand at 35°C for exactly 10 minutes, then add exactly 0.5 mL of 1 mol/L hydrochloric acid TS, and immediately shake. Determine the absorbance under Ultraviolet-visible Spectrophotometry <2.24>, A_T, of this solution at 640 nm, using water as the blank. Determine the absorbances, A_{S1} and A_{S2}, of the solutions obtained with the standard solution (1) and the standard solution (2) in the same manner as the sample solution.

Amount [mg (potency)] of lysozyme per mg, calculated on the dried basis

$$= (W_S/2W_T) \times \{(A_{S1} - A_T)/(A_{S1} - A_{S2}) + 1\}$$

W_S: Amount (mg) of Lysozyme Reference Standard, calculated on the dried basis.
W_T: Amount (mg) of the sample, calculated on the dried basis.

Containers and storage Containers—Tight containers.

Macrogol 400

Polyethylene Glycol 400

マクロゴール 400

Macrogol 400 is a polymer of ethylene oxide and water, represented by the formula HOCH₂(CH₂OCH₂)_nCH₂OH, in which the value of n ranges from 7 to 9.

Description Macrogol 400 occurs as a clear, colorless and viscous liquid. It has no odor or a slight, characteristic odor. It is miscible with water, with methanol, with ethanol (95) and with pyridine. It is soluble in diethyl ether. It is slightly hygroscopic. Congealing point: 4 – 8°C Specific gravity d₂₀²⁰: 1.110 – 1.140

Identification Dissolve 0.05 g of Macrogol 400 in 5 mL of dilute hydrochloric acid, add 1 mL of barium chloride TS, shake, and filter, if necessary. To the filtrate add 1 mL of a solution of phosphomolybdic acid n-hydrate (1 in 10): a yellow-green precipitate is formed.

pH <2.54> Dissolve 1.0 g of Macrogol 400 in 20 mL of water: the pH of this solution is between 4.0 and 7.0.

Purity (1) Acidity—Dissolve 5.0 g of Macrogol 400 in 20 mL of neutralized ethanol, and add 2 drops of phenolphthalein TS and 0.20 mL of 0.1 mol/L sodium hydroxide VS: the solution is red in color.

(2) Ethylene glycol and diethylene glycol—Dissolve 4.0 g of Macrogol 400 in water to make exactly 10 mL, and use this solution as the sample solution. Weigh accurately about 50 mg each of ethylene glycol and diethylene glycol, dissolve in water to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 2 μ L each of the sample solution and standard solution as directed under Gas Chromatography <2.02> according to the following conditions. Determine the peak heights, H_{Ta} and H_{Sa} , of ethylene glycol of each solution, and the peak heights, H_{Tb} and H_{Sb} , of diethylene glycol, and calculate the amount of ethylene glycol and diethylene glycol: the sum of the contents of ethylene glycol and diethylene glycol is not more than 0.25%.

$$\begin{aligned} &\text{Amount (mg) of ethylene glycol} \\ &= W_{Sa} \times (H_{Ta}/H_{Sa}) \times (1/10) \end{aligned}$$

$$\begin{aligned} &\text{Amount (mg) of diethylene glycol} \\ &= W_{Sb} \times (H_{Tb}/H_{Sb}) \times (1/10) \end{aligned}$$

W_{Sa} : Amount (mg) of ethylene glycol for gas chromatography

W_{Sb} : Amount (mg) of diethylene glycol for gas chromatography

Operating conditions—

Detector: A hydrogen flame-ionization detector.

Column: A column about 3 mm in inside diameter and about 1.5 m in length, packed with siliceous earth for gas chromatography, 150 to 180 μ m in particle diameter, coated with D-sorbitol at the ratio of 12%.

Column temperature: A constant temperature of about 165 °C.

Carrier gas: Nitrogen or helium.

Flow rate: Adjust the flow rate so that the retention time of diethylene glycol is about 8 minutes.

Selection of column: Proceed with 2 μ L of the standard solution under the above operating conditions, and calculate the resolution. Use a column clearly dividing peaks of ethylene glycol and diethylene glycol in this order.

Detection sensitivity: Adjust the detection sensitivity so that the peak height of diethylene glycol obtained from 2 μ L of the standard solution composes about 80% of the full scale.

Average molecular mass Add 42 g of phthalic anhydride to 300 mL of freshly distilled pyridine, exactly measured, in a 1-L light-resistant glass-stoppered bottle. Shake the bottle vigorously to dissolve the solid, and allow to stand for 16 hours or more. Pipet 25 mL of this solution into an about 200-mL glass-stoppered pressure bottle. Add about 1.5 g of Macrogol 400, accurately weighed, stopper the bottle, wrap it securely with strong cloth, and immerse in a water bath, having a temperature of $98 \pm 2^\circ\text{C}$, to the level so that the mixture in the bottle soaks completely in water. Maintain the temperature of the bath at $98 \pm 2^\circ\text{C}$ for 30 minutes. Remove the bottle from the bath, and allow to cool in air to room temperature. Add exactly 50 mL of 0.5 mol/L sodium hydroxide VS and 5 drops of a solution of phenolphthalein in pyridine (1 in 100). Titrate <2.50> with 0.5 mol/L sodium hydroxide VS until a light red color remains for not less than 15 seconds. Perform a blank determination.

$$\begin{aligned} &\text{Average molecular mass} \\ &= (W \times 4000)/(a - b) \end{aligned}$$

W : Amount (g) of sample.

a : Volume (mL) of 0.5 mol/L sodium hydroxide VS used in the blank determination.

b : Volume (mL) of 0.5 mol/L sodium hydroxide VS used in the test of the sample.

Average molecular mass is between 380 and 420.

Water <2.48> Not more than 1.0% (2 g, direct titration).

Residue on ignition <2.44> Not more than 0.1% (1 g).

Containers and storage Containers—Tight containers.

Macrogol 1500

Polyethylene Glycol 1500

マクロゴール 1500

Macrogol 1500 is a mixture containing equal amounts of lower and higher polymers of ethylene oxide and water, represented by the formula $\text{HOCH}_2(\text{CH}_2\text{OCH}_2)_n\text{CH}_2\text{OH}$, in which the value of n is 5 or 6 for the lower polymers and from 28 to 36 for the higher.

Description Macrogol 1500 occurs as a white, smooth petrolatum-like solid. It is odorless or has a faint, characteristic odor.

It is very soluble in water, in pyridine and in diphenyl ether, freely soluble in methanol, sparingly soluble in ethanol (95), very slightly soluble in ethanol (99.5), and practically insoluble in diethyl ether.

Congeeing point: $37 - 41^\circ\text{C}$

Identification Dissolve 0.05 g of Macrogol 1500 in 5 mL of dilute hydrochloric acid, add 1 mL of barium chloride TS, shake, and filter, if necessary. To the filtrate add 1 mL of a solution of phosphomolybdic acid n -hydrate (1 in 10): a yellow-green precipitate is formed.

pH <2.54> Dissolve 1.0 g of Macrogol 1500 in 20 mL of water: the pH of the solution is between 4.0 and 7.0.

Purity (1) Clarity and color of solution—Dissolve 5.0 g of Macrogol 1500 in 50 mL of water: the solution is clear and colorless.

(2) Acidity—Dissolve 5.0 g of Macrogol 1500 in 20 mL of neutralized ethanol, and add 2 drops of phenolphthalein TS and 0.20 mL of 0.1 mol/L sodium hydroxide VS: the solution is red in color.

(3) Ethylene glycol and diethylene glycol—Place 50.0 g of Macrogol 1500 in a distilling flask, add 75 mL of diphenyl ether, warm to dissolve if necessary, distil slowly under a reduced pressure of 0.13 to 0.27 kPa and take 25 mL of the distillate in a 100-mL container with 1-mL graduation. To the distillate add exactly 20 mL of water, shake vigorously, cool in ice water, congeal the diphenyl ether, and filtrate into a 25-mL volumetric flask. Wash the residue with 5.0 mL of ice-cold water, combine the washings with the filtrate, warm to room temperature, and add water to make 25 mL. Transfer this solution to a glass-stoppered flask, shake with 25.0 mL of freshly distilled acetonitrile, and use this solution as the sample solution. Separately, to 62.5 mg of diethylene glycol

add a mixture of water and freshly distilled acetonitrile (1:1) to make exactly 25 mL, and use this solution as the standard solution. Take exactly 10 mL each of the sample solution and the standard solution, and add to each exactly 15 mL of cerium (IV) diammonium nitrate TS. Perform the test with this solution as directed under Ultraviolet-visible Spectrophotometry <2.24> within 2 to 5 minutes: the absorbance of the solution obtained from the sample solution at the wavelength of maximum absorption at about 450 nm is not larger than the absorbance of the solution obtained from the standard solution.

Water <2.48> Not more than 1.0% (2 g, direct titration).

Residue on ignition <2.44> Not more than 0.1% (1 g).

Containers and storage Containers—Tight containers.

Macrogol 4000

Polyethylene Glycol 4000

マクロゴール 4000

Macrogol 4000 is a polymer of ethylene oxide and water, represented by the formula $\text{HOCH}_2(\text{CH}_2\text{OCH}_2)_n\text{CH}_2\text{OH}$, in which the value of n ranges from 59 to 84.

Description Macrogol 4000 is a white, paraffin-like solid, occurring as flakes or powder. It is odorless or has a faint, characteristic odor.

It is very soluble in water, freely soluble in methanol and in pyridine, and practically insoluble in ethanol (99.5) and in diethyl ether.

Congealing point: 53 – 57°C

Identification Dissolve 0.05 g of Macrogol 4000 in 5 mL of dilute hydrochloric acid, add 1 mL of barium chloride TS, shake, and filter, if necessary. To the filtrate add 1 mL of a solution of phosphomolybdic acid n -hydrate (1 in 10): a yellow-green precipitate is formed.

pH <2.54> Dissolve 1.0 g of Macrogol 4000 in 20 mL of water: the pH of this solution is between 4.0 and 7.5.

Purity (1) Clarity and color of solution—A solution of 5.0 g of Macrogol 4000 in 50 mL of water is clear and colorless.

(2) Acidity—Dissolve 5.0 g of Macrogol 4000 in 20 mL of neutralized ethanol by warming, cool, and add 0.20 mL of 0.1 mol/L sodium hydroxide VS and 1 drop of phenolphthalein TS: the color of the solution is red.

Average molecular mass Weigh accurately about 12.5 g of Macrogol 4000, transfer to an about 200-mL glass-stoppered pressure bottle, add about 25 mL of pyridine, dissolve by warming, and allow to cool. Separately, pipet 300 mL of freshly distilled pyridine into a 1000-mL light-resistant, glass-stoppered bottle, add 42 g of phthalic anhydride, dissolve with vigorous shaking, and allow to stand for 16 hours or more. Pipet 25 mL of this solution, transfer to the former pressure bottle, stopper the bottle tightly, wrap it securely with strong cloth, and immerse in a water bath, previously heated at $98 \pm 2^\circ\text{C}$, to the level so that the mixture in the

bottle soaks completely in water. Maintain the temperature of the bath at $98 \pm 2^\circ\text{C}$ for 30 minutes. Remove the bottle from the bath, and allow to cool in air to room temperature. Add exactly 50 mL of 0.5 mol/L sodium hydroxide VS and 5 drops of a solution of phenolphthalein in pyridine (1 in 100). Titrate <2.50> with 0.5 mol/L sodium hydroxide VS until a light red color remains for not less than 15 seconds. Perform a blank determination.

$$\begin{aligned} &\text{Average molecular mass} \\ &= (W \times 4000)/(a - b) \end{aligned}$$

W : Amount (g) of sample.

a : Volume (mL) of 0.5 mol/L sodium hydroxide VS consumed in the blank determination.

b : Volume (mL) of 0.5 mol/L sodium hydroxide VS consumed in the test of the sample.

Average molecular mass is between 2600 and 3800.

Water <2.48> Not more than 1.0% (2 g, direct titration).

Residue on ignition <2.44> Not more than 0.2% (1 g).

Containers and storage Containers—Well-closed containers.

Macrogol 6000

Polyethylene Glycol 6000

マクロゴール 6000

Macrogol 6000 is a polymer of ethylene oxide and water, represented by the formula $\text{HOCH}_2(\text{CH}_2\text{OCH}_2)_n\text{CH}_2\text{OH}$, in which the value of n ranges from 165 to 210.

Description Macrogol 6000 is a white, paraffin-like solid, occurring as flakes or powder. It is odorless or has a faint, characteristic odor.

It is very soluble in water, freely soluble in pyridine, and practically insoluble in methanol, in ethanol (95), in ethanol (99.5) and in diethyl ether.

Congealing point: 56 – 61°C

Identification Dissolve 0.05 g of Macrogol 6000 in 5 mL of dilute hydrochloric acid, add 1 mL of barium chloride TS, shake, and filter, if necessary. To the filtrate add 1 mL of a solution of phosphomolybdic acid n -hydrate (1 in 10): a yellow-green precipitate is formed.

pH <2.54> Dissolve 1.0 g of Macrogol 6000 in 20 mL of water: the pH of this solution is between 4.5 and 7.5.

Purity (1) Clarity and color of solution—Dissolve 5.0 g of Macrogol 6000 in 50 mL of water: the solution is clear and colorless.

(2) Acidity—Dissolve 5.0 g of Macrogol 6000 in 20 mL of neutralized ethanol by warming, cool, and add 0.20 mL of 0.1 mol/L sodium hydroxide VS and 1 drop of phenolphthalein TS: the color of the solution is red.

Average molecular mass Weigh accurately about 12.5 g of Macrogol 6000, transfer to an about 200-mL glass-stoppered pressure bottle, add about 25 mL of pyridine, dissolve by warming, and allow to cool. Separately, pipet 300 mL of

freshly distilled pyridine into a 1000-mL light-resistant, glass-stoppered bottle, add 42 g of phthalic anhydride, dissolve with vigorous shaking, and allow to stand for 16 hours or more. Pipet 25 mL of this solution, transfer to the former pressure bottle, stopper the bottle tightly, wrap it securely with strong cloth, and immerse in a water bath, previously heated at $98 \pm 2^\circ\text{C}$, to the level so that the mixture in the bottle soaks completely in water. Maintain the temperature of the bath at $98 \pm 2^\circ\text{C}$ for 30 minutes. Remove the bottle from the bath, and allow to cool in air to room temperature. Add exactly 50 mL of 0.5 mol/L sodium hydroxide VS and 5 drops of a solution of phenolphthalein in pyridine (1 in 100). Titrate <2.50> with 0.5 mol/L sodium hydroxide VS until a light red color remains for not less than 15 seconds. Perform a blank determination in the same manner.

$$\begin{aligned} \text{Average molecular mass} \\ = (W \times 4000)/(a - b) \end{aligned}$$

W: Amount (g) of sample.

a: Volume (mL) of 0.5 mol/L sodium hydroxide VS consumed in the blank determination.

b: Volume (mL) of 0.5 mol/L sodium hydroxide VS consumed in the test of the sample.

Average molecular mass is between 7300 and 9300.

Water <2.48> Not more than 1.0% (2 g, direct titration).

Residue on ignition <2.44> Not more than 0.2% (1 g).

Containers and storage Containers—Well-closed containers.

Macrogol 20000

Polyethylene Glycol 20000

マクロゴール 20000

Macrogol 20000 is a polymer of ethylene oxide and water, represented by the formula $\text{HOCH}_2(\text{CH}_2\text{OCH}_2)_n\text{CH}_2\text{OH}$, in which the value of *n* lies between 340 and 570.

Description Macrogol 20000 occurs as white, paraffin-like flakes or powder. It is odorless or has a faint, characteristic odor.

It is freely soluble in water and in pyridine, and practically insoluble in methanol, in ethanol (95), in anhydrous diethyl ether, in petroleum benzine and in macrogol 400.

Congealing point: $56 - 64^\circ\text{C}$

Identification Dissolve 0.05 g of Macrogol 20000 in 5 mL of dilute hydrochloric acid, add 1 mL of barium chloride TS, shake, and filter, if necessary. To the filtrate add 1 mL of a solution of phosphomolybdic acid *n*-hydrate (1 in 10): a yellow-green precipitate is formed.

pH <2.54> Dissolve 1.0 g of Macrogol 20000 in 20 mL of water: the pH of this solution is between 4.5 and 7.5.

Purity (1) Clarity and color of solution—Dissolve 5.0 g of Macrogol 20000 in 50 mL of water: the solution is clear and colorless.

(2) Acidity—Dissolve 5.0 g of Macrogol 20000 in 20 mL

of neutralized ethanol by warming, cool, and add 0.20 mL of 0.1 mol/L sodium hydroxide VS and 1 drop of phenolphthalein TS: the color of the solution is red.

Average molecular mass Weigh accurately about 15 g of Macrogol 20000, transfer to an about 200-mL glass-stoppered pressure bottle, add about 25 mL of pyridine, dissolve by warming, and allow to cool. Separately, pipet 300 mL of freshly distilled pyridine into a 1000-mL light-resistant glass-stoppered bottle, add 42 g of phthalic anhydride, dissolve with vigorous shaking, and allow to stand for 16 hours or more. Pipet 25 mL of this solution, transfer to the former pressure bottle, stopper the bottle tightly, wrap it securely with strong cloth, and immerse in a water bath, having a temperature of $98 \pm 2^\circ\text{C}$, to the same depth as the mixture in the bottle. Maintain the temperature of the bath at $98 \pm 2^\circ\text{C}$ for 60 minutes. Remove the bottle from the bath, and allow to cool in air to room temperature. Add exactly 50 mL of 0.5 mol/L sodium hydroxide VS and 5 drops of a solution of phenolphthalein in pyridine (1 in 100). Titrate <2.50> with 0.5 mol/L sodium hydroxide VS until a light red color remains for not less than 15 seconds. Perform a blank determination.

$$\begin{aligned} \text{Average molecular mass} \\ = (W \times 4000)/(a - b) \end{aligned}$$

W: Amount (g) of sample.

a: Volume (mL) of 0.5 mol/L sodium hydroxide VS used in the blank determination.

b: Volume (mL) of 0.5 mol/L sodium hydroxide VS used in the test of the sample.

Average molecular mass is between 15000 and 25000.

Water <2.48> Not more than 1.0% (2 g, direct titration).

Residue on ignition <2.44> Not more than 0.2% (1 g).

Containers and storage Containers—Well-closed containers.

Macrogol Ointment

Polyethylene Glycol Ointment

マクロゴール軟膏

Method of preparation

Macrogol 4000	500 g
Macrogol 400	500 g
To make 1000 g	

Melt Macrogol 4000 and Macrogol 400 by warming on a water bath at 65°C , and mix well until it congeals. Less than 100 g of Macrogol 4000 or Macrogol 400 may be replaced by an equal amount of Macrogol 400 or Macrogol 4000 to prepare 1000 g of a proper soft ointment.

Description Macrogol Ointment is white in color. It has a faint, characteristic odor.

Identification Dissolve 0.05 g of Macrogol Ointment in 5 mL of dilute hydrochloric acid, add 1 mL of barium chloride TS, shake, filter if necessary, and add 1 mL of a solution of phosphomolybdic acid *n*-hydrate (1 in 10) to the filtrate: a yellow-green precipitate is formed.