

Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES

E. _____ S. _____

Re: Expediente No. 06.073.023

Solicitud de patente de invención titulada:

**“CONSERVANTES ANTIMICROBIANOS PARA LOGRAR UNA
FORMULACION DE DOSIS MULTIPLES USANDO CICLODEXTRINAS B
PARA FORMAS DE DOSIFICACION LIQUIDAS”.**

Solicitante: PFIZER PRODUCTS INC.

Nuestra Ref.: P2006/000126

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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RESPUESTA AL OFICIO No. 10382 NOTIFICADO POR FIJACIÓN EN LISTA No. 276
DE FECHA 13 DE AGOSTO DE 2010
(SEGUNDO EXAMEN DE FONDO)

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad solicitante, me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del plazo legal previsto en el artículo 45 de la Decisión 486, en los siguientes términos:

1. a- El Examinador argumenta que las patentes US2003/0139443 (D1) y WO03/080079 (D2), afectan el nivel inventivo de la presente invención.

El Examinador señala que D1, el estado de la técnica más cercano a la presente invención, revela composiciones líquidas para administración por vía parenteral, que comprenden un antagonista del receptor NK-1 (o sus sales), un preservativo (PEG), un vehículo (glicerina) y una β -ciclodextrina (sulfobutileter SBE ciclodextrina), diferenciándose de la presente invención únicamente en la presencia de metacresol.

El Examinador considera que los efectos alcanzados con el uso de metacresol (buena estabilidad con eficacia antimicrobiana frente a los microorganismos probados y buena solubilidad) eran de esperarse.

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De otra parte, el Examinador argumenta que D2 enseña una formulación líquida con pH ácido que comprende sulfobutiléter ciclodextrina complejada con principios activos con el fin de aumentar su solubilidad y estabilidad, que además, pueden contener metacresol. Adicionalmente, las composiciones farmacéuticas de D2 presentan actividad antimicrobiana contra los mismos microorganismos para los que es efectiva la presente solicitud, y en donde la ciclodextrina no reduce la actividad preservante del metacresol, que es el mismo efecto técnico alcanzado por las formulaciones de la presente invención.

Por último, el Examinador señala que el Capítulo Descriptivo de la presente solicitud afirma que la formulación reivindicada se logró como resultado de la optimización de la afinidad entre la ciclodextrina y el fármaco versus el conservante (como –según el Examinador- supuestamente ocurre en D2). El Examinador considera tal optimización una labor en la rutina diaria del técnico medio en la materia.

El Examinador concluye que una persona medianamente versada en la materia, enfrentando el problema de cómo obtener composiciones alternativas de un compuesto de Fórmula 1, con mayor solubilidad y eficacia antimicrobiana, habría incluido el metacresol enseñado en D2 en las composiciones divulgadas en D1, y así habría alcanzado de manera obvia las composiciones objeto de la presente invención.

b- i) En primer lugar, el Examinador aparentemente no ha comprendido claramente la naturaleza de la presente invención, en qué consiste la composición farmacéutica reivindicada, ni sus diferencias con las composiciones del arte previo.

Como lo mencioné en mi respuesta al primer examen de fondo, D1 divulga una formulación que contiene antagonistas del receptor de NK-1 y β -ciclodextrina, sin enseñar o incluso sugerir el uso de un preservativo.

De otra parte, D2 enseña el uso de sulfoalquiléter ciclodextrinas como preservativos en una formulación. En D2, el uso de las ciclodextrinas está destinado exclusivamente a reducir la actividad de agua de la formulación, hasta valores en los que los microorganismos de interés son incapaces de crecer y/o mueren (ver página 11, líneas 3 a 7). Por lo tanto, la cantidad de ciclodextrina en la formulación divulgada en D2 tiene como única función la preservación de la misma durante el tiempo de almacenamiento (ver página 11, líneas 17 a 19).

Adicionalmente, difiero respetuosamente de la posición del Examinador cuando argumenta que en las formulaciones divulgadas en D2 la sulfobutiléter ciclodextrina se encuentra acomplejada

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con principios activos con el fin de aumentar su solubilidad y estabilidad. Por el contrario, D2 es claro en especificar que la ciclodextrina allí incluida no necesariamente se encuentra complejada con el principio activo (ver página 11, líneas 21 y 22). De hecho, el efecto técnico brindado por D2 es que la presencia de la ciclodextrina hace que el uso de un preservante no sea necesario (ver página 30, líneas 32 y 33). En conclusión, el principal objetivo de D2 es proporcionar composiciones en donde el efecto preservante se obtiene gracias a la ciclodextrina, sin la necesidad de un preservante adicional.

Teniendo en cuenta lo anterior, la persona medianamente versada en la materia, siguiendo las enseñanzas de D2, necesariamente se habría alejado de la presente invención. De hecho, contrario a lo divulgado en D2, la presente invención:

- provee composiciones que comprende un ingrediente activo, una ciclodextrina y un preservativo farmacéuticamente aceptable;
- busca mejorar la solubilidad del ingrediente activo, mejorando también la tolerancia en el sitio de inyección;
- proporciona mejoras en la solubilidad, obtenidas gracias al efecto de la complejación del activo con la ciclodextrina;
- supera el problema de la complejación entre los agentes preservantes y la ciclodextrina que produce ineffectividad antimicrobiana, pues la ciclodextrina se compleja tanto con ingredientes activos como con preservantes antimicrobianos, afectando así la estabilidad, la eficacia y la efectividad de los preservativos;
- determina la afinidad entre el ingrediente activo y la ciclodextrina, que debe ser mayor a la afinidad entre el preservante y dicha ciclodextrina (preferiblemente mayor que o igual a 10 veces).

Ahora bien, habiendo establecido lo divulgado en D2 y la presente invención, es claro que la persona medianamente versada en la materia siguiendo dichas enseñanzas habría tratado, en el mejor de los casos, de elaborar una composición en donde la ciclodextrina no tuviera el propósito de solubilizar el ingrediente activo, sino más bien de preservar la misma mediante la reducción de la actividad de agua. De hecho, D2 directamente lleva a evitar la inclusión de un preservante.

En este punto quisiera llamar la atención del Examinador al Ejemplo 1 de D2, en donde la Formulación 3 incluye metabisulfito de sodio como un agente preservante. Los resultados ilustrados en las Tablas 1 a 14 muestran que las composiciones que sólo contienen ciclodextrina y el ingrediente activo (incluso sin el agente preservante), proporcionan reducción del crecimiento microbiano y efectos preservantes, durante por lo menos 48 horas. Es más, las Tablas 3 y 12 a 14

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muestran que los resultados mejorados del efecto antimicrobiano se obtienen con composiciones en donde se excluye un agente preservante.

ii) De otra parte, es importante resaltar que, como lo establecen las directrices del Manual Andino de Patentes (MAP), “la cuestión para el Examinador es si la invención reivindicada es o no evidente para un técnico en la materia”¹. Adicionalmente, en el análisis de nivel inventivo el Examinador debe proporcionar argumentos claros e inequívocos que expliquen las motivaciones expresamente contenidas en el arte previo que necesariamente conduzcan a la persona medianamente versada en la materia a la invención reivindicada², sin necesidad de experimentación adicional alguna, y con una expectativa de éxito indudable sobre los resultados a alcanzar.

En el presente caso, el Examinador presume que una persona medianamente versada en la materia habría incluido las enseñanzas de D2 en las formulaciones divulgadas en D1, sin tener en cuenta que, de acuerdo con las sugerencias y enseñanzas allí incluidas, tal y como se ha expuesto, el técnico medio habría excluido un preservante de las formulaciones, ya que la ciclodextrina cumpliría esa función, y, adicionalmente, no habría encontrado motivación alguna para determinar la afinidad necesaria entre la ciclodextrina y el preservante, y la ciclodextrina y el ingrediente activo, para evitar el problema creado en la estabilidad, eficacia y la efectividad de los preservativos complejados.

Adicionalmente, difiero respetuosamente de la posición del Examinador cuando establece que la presente solicitud es obvia a la luz de lo divulgado en D1 y D2, ya que, como lo ha determinado el Tribunal Andino de Justicia, “lo obvio es aquello que salta a la vista de manera clara y directa; lo “que se encuentra o pone delante de los ojos, muy claro o que no tiene dificultad”. Lo evidente consiste en la “certeza clara y manifiesta de la que no se puede dudar, cierto, claro, patente y sin la menor duda”. Continúa el Tribunal diciendo que “una invención goza de nivel inventivo cuando a los ojos de un experto medio en el asunto de que se trate, se necesita algo más que la simple aplicación de los conocimientos técnicos en la materia para llegar a ella, es decir, que de conformidad con el estado de la técnica el invento no es consecuencia clara y directa de dicho estado.”³

¹ Manual Andino de Patentes. OMPI, EPO, Secretaría de la Comunidad Andina, 2004. ISBN9978-43-855-6. Sección 10.1.

² Manual Andino de Patentes. OMPI, EPO, Secretaría de la Comunidad Andina, 2004. ISBN9978-43-855-6. Secciones 10.2.1. Página 77.

³ Proceso 10-IP-2009, página 12, párrafos 5 y 6.

En el presente caso, nada en D1 y/o D2 motivaría a la persona medianamente versada en la materia a usar un preservante en una formulación que contiene ciclodextrina y un ingrediente activo. Por el contrario, el técnico medio, siguiendo lo enseñado en D1 y/o D2 habría evitado definitivamente el uso de un agente preservante.

Finalmente, es importante anotar que la persona medianamente versada en la materia con el objetivo de solucionar el problema de formular compuestos antagonistas del receptor NK-1 de Fórmula I, incluyendo una β -ciclodextrina –con el fin de mejorar la solubilidad– no habría encontrado sugerencia alguna en D1 y/o D2 sobre cómo evitar el inconveniente de la complejación de los preservantes y las β -ciclodextrinas, y adicionalmente, superar la ineffectividad antimicrobiana. Nada en el arte previo provee enseñanzas o sugerencia que puedan conducir al técnico medio a determinar el balance óptimo entre una concentración suficiente de ciclodextrina (por ejemplo SBE-CD, proporcionando una tolerancia en el sitio de inyección aceptable) y el preservante antimicrobiano (por ejemplo metacresol).

Adicionalmente, como lo mencioné en mi respuesta al primer examen de fondo, es bien conocido en el campo de las formulaciones farmacéuticas que la ciclodextrina se compleja tanto con ingredientes activos como con preservantes antimicrobianos, afectando así la estabilidad, la eficacia y la efectividad de los preservativos. Otros documentos del arte previo no citados por el Examinador, enseñan que los preservativos a base de fenol halogenado no causan precipitación del complejo de inclusión farmaco/ciclodextrina/preservante (EP0119737). Adicionalmente, el documento WO00/12137 revela que los preservantes típicos se complejan con ciclodextrinas y por lo tanto sugiere emplear dióxido de cloro (chlorine dioxide), ácido sórbico, sorbatos de metales alcalinos, a concentraciones cercanas al 1% (p/v) como preservantes, los cuales no forman complejos con el componente de ciclodextrina. Una tercera referencia, Ono, et.al. (ANEXO 1), se refiere a la complejación en el uso de ciclodextrinas, particularmente en lo relativo a la solubilidad y la penetración de la fenacetina y varios ácidos benzoicos. Como se observa en Ono, et. al., para modelar relaciones de complejación se requiere, por lo menos de la determinación de la estabilidad y las constantes de velocidad de penetración de las fracciones libres y complejadas. Es más, según Ono, et. al., las tasas de penetración de los complejos de ciclodextrina se ven significativamente afectadas por la presencia de segundas moléculas (como un preservante), debido a la competencia de inclusión.

Así las cosas, una persona versada en la materia con la información disponible en el estado del arte a la fecha de presentación de la invención se vería motivada a excluir de formulaciones que contienen ciclodextrina, aquellos preservantes diferentes a fenoles halogenados y solamente a incluir aquellos sugeridos en el documento WO00/12137. Nada en D1 o en el estado del arte

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sugiere el empleo de preservativos típicos como el metacresol en una composición que comprende β -ciclodextrinas y un compuesto antagonista NK1 de Formula I.

En conclusión, el arte previo demuestra claramente, que debido a las propiedades fisicoquímicas y biológicas de un fármaco dado, las constantes de estabilidad, las constantes de penetración, de velocidad, y la estequiometría competitiva de complejación para fármacos activos, preservativos y excipientes, la persona medianamente versada en la materia no estaría en capacidad de determinar la composición farmacéutica de la presente invención, previendo los resultados alcanzados con una razonable expectativa de éxito y sin experimentación indebida adicional.

2. Por último, deseo reiterar que hasta el momento han sido concedidas patentes de invención en muchas legislaciones, protegiendo la materia reivindicada en la presente solicitud, como por ejemplo en Sudáfrica (2006/05148), Australia (2005216709), Canadá (2554346), Korea del Sur (834232), Rusia (2332997) y Singapur (P-124084).

Si bien es cierto que las concesiones de patentes en oficinas de otros países no son vinculantes en el sentido que obliguen a la SIC a tomar una decisión en el mismo sentido, considero que dichas concesiones sugieren el cumplimiento de los requisitos de patentabilidad de la materia reivindicada, ya que los criterios de evaluación y estudio aplicados para concluir la aceptación de dichas patentes resultan equivalentes a los que la SIC debe emplear.

3. Por todo lo anterior, considero que las objeciones presentadas por el Examinador en su examen de fondo No. 2517 han sido resueltas y por consiguiente, solicito respetuosamente se tengan por cumplidos los requisitos de patentabilidad en tanto no se desvirtuó ninguno de ellos, y se proceda con la concesión de la patente.

Atentamente,



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Anexo 1: Ono N. et al. "Determination of stability constant of β -cyclodextrin complexes using the membrane permeation technique and the permeation behavior of drug-competing agent β -cyclodextrin ternary systems". European Journal of Pharmaceutical Sciences. Pages 133-139 (1999)

Determination of stability constant of β -cyclodextrin complexes using the membrane permeation technique and the permeation behavior of drug–competing agent– β -cyclodextrin ternary systems

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Abstract

The stability constants (K_c) of β -cyclodextrin (β -CyD) complexes with phenacetin and various benzoic acids were determined using the membrane permeation technique using a cellophane membrane permeable for guest molecules which was impermeable for β -CyD molecules. The permeation rate equations of guests in the presence of β -CyD in the donor phase were derived and the permeation profiles were analyzed as a function of time to determine the K_c values. The K_c values determined using the membrane permeation technique were in close agreement with those determined by the conventional kinetic method and the solubility method. The membrane permeation technique is of greater advantage than the conventional methods, because in the former method the stability constant can be obtained from only one experimental run by analyzing the permeation data as a function of time. In the latter method, on the other hand, some property changes have to be measured at various CyD concentrations and in turn analyzed as a function of CyD concentration. The permeation profiles of phenacetin of the drug–competing agent– β -CyD ternary system were estimated by using the stability constants and the experimental curves closely matched the theoretically derived ones. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: β -Cyclodextrin; Inclusion complex; Stability constant; Membrane permeation

1. Introduction

Cyclodextrins (CyDs) are known to form inclusion complexes with various drug molecules and are utilized successfully for improvement of drug properties such as solubility, stability and bioavailability, etc. (Uekama et al., 1998). Since the changes of physicochemical and biological properties of a drug are dependent on the magnitude of the stability constant of CyD complexes, it is important for prediction or simulation of the property change to determine accurately this parameter (Hirayama and Uekama, 1987). There are many methods for determination of stability constants of CyD complexes, using techniques such as solubility (Higuchi and Connors, 1965), potentiometry (Miyaji et al., 1976), high-pressure liquid chromatography (Uekama et al., 1978), and kinetic (Bender and Komiyama, 1978) and spectroscopic (Cramer et al., 1967; Thakkar et al., 1972) methods. These methods are generally based on the following: (1) The titration of a certain averaged chemical or physical property of a guest

molecule with CyDs or vice versa and in turn the analysis of the CyD concentration dependence by means of the Benesi-Hildebrand equation (Benesi and Hildebrand, 1949), the Lineweaver-Burk equation (Lineweaver and Burk, 1934) or their modified forms (Guttman, 1962). (2) The direct determination of free or complexed guest or host molecule. If the free component is separated from CyD solutions, we can estimate directly the free concentration and then determine the stability constant. For instance, Saito et al. (1998) employed static head-space gas chromatography for determining the stability constants of 2-hydroxypropyl- β -CyD complexes with volatile fragrances, assuming that only the free guest is in an equilibrium between gas and liquid phases whereas the complexed component is non-volatile. Therefore, the fragrance concentration in the head-space corresponds to that of the free component. In the case of protein binding experiments (Martin et al., 1983), the free fraction of a drug is separated from the protein-bound drug through a cellophane membrane and then the binding constant is calculated. Sideris et al. (1994) developed the automated flow-injection serial dynamic dialysis technique for determination of stability constants of CyD complexes. In

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special cases of slow inclusion equilibrium, nuclear magnetic resonance peaks of the free and complexed guest or host molecules are separately observed and in turn their concentrations can be determined from each signal intensity or area (Saito et al., 1990).

While simulating on intestinal absorption of CyD complexes using an artificial membrane permeable for drugs but impermeable for CyDs, we observed that the permeation rate of drugs is dependent not only on the CyD concentration but also on the time of the permeation. This may be due to the continuous change of the inclusion equilibrium in a donor phase, because the concentration of drug decreases due to the permeation into an acceptor, whereas that of CyDs is constant. Therefore, the permeation profile of the drug in the presence of CyDs in the donor phase has to implicate information of not only the permeation rate but also the stability constant of the complex. Incorporating this idea, we developed a new method for determination of the stability constant of β -CyD complexes, by analyzing the permeation profile as a function of time. Furthermore, the membrane permeation behavior of a drug from the ternary system, i.e., in the presence of both CyDs and competing agents, was simulated to justify the validity of the method and to gain an insight into the permeation mechanism of drugs. Phenacetin and benzoic acid derivatives were employed as the model drug and competing agents, respectively, because they are known to form 1:1 inclusion complexes with β -CyD (Lach and Chin, 1964; Connors and Lipari, 1976).

2. Experimental procedures

2.1. Materials

CyDs were supplied from Japan Maize Co. (Tokyo, Japan) and recrystallized from water. The following chemicals were used after recrystallization from methanol-water: phenacetin (Sigma-Aldrich, USA), toluic acids (Wako Pure Chemicals Co., Tokyo, Japan), chloro-, bromo- and iodo-benzoic acids (Nakalai Tesque or Tokyo Kasei, Tokyo, Japan). All other chemicals and solvents were of analytical reagent grade, and deionized double-distilled water was used throughout the study.

2.2. Permeation studies

Permeation behavior of phenacetin and benzoic acids through a cellophane membrane (Spectra/Por® MWCO 500, SPECTRUM, Houston, USA) was examined using the side-by-side type permeation cell apparatus, as reported previously (Uckama et al., 1980; Ohki et al., 1980). The cellophane membrane was selected because it is less adsorptive of benzoic acids compared with that of ultrafiltration membranes such as Amicon Diaflow® YC06 and

YM1 and because of its impermeability for β -CyD under the present experimental conditions (permeation of β -CyD for 6 h: <0.04% through Spectra/Por® MWCO 500, whereas about 3% through Spectra/Por® MWCO 1000 and 2000). The sample solution (generally 1.0×10^{-4} M drugs and 1.0×10^{-2} M β -CyD in 10 mM phosphate buffer (pH 7.4, 55 ml) was put into the donor cell compartment while the same volume of the phosphate buffer solution without samples was placed in the acceptor cell compartment. Both solutions in the permeation cells were stirred using a stainless-steel bar at 100 rpm at 37°C. At appropriate intervals an aliquot (0.2 ml) was pipetted from the donor and acceptor cell compartments and the drug concentrations were measured using high-performance liquid chromatography (HPLC) under the following conditions. An equal volume of the phosphate buffer was added to maintain a constant volume of the medium HPLC condition: a Hitachi 655A-11 HPLC chromatograph and a Hitachi 655A UV monitor (Tokyo, Japan), a YMC ODS AM-303-S-5 column (4.6 I.D. $\times$ 250 mm, Kyoto, Japan), a mobile phase of 150 mM phosphoric acid-acetonitrile (1:1 v/v), a flow-rate of 1.0 ml min⁻¹, a detection of 240 nm. The concentration of β -CyD in both cells was determined according to the method of Koizumi et al. (1985). The experiments were replicated generally three times and the stability constants were expressed as a mean $\pm$ standard deviation (S.D.).

2.3. Solubility studies

The solubility method was carried out according to the method of Higuchi and Connors (Higuchi and Connors, 1965). The screw capped vials containing samples (10.0–70.0 mg) in excess amounts in aqueous β -CyD solutions (3.0 ml of 0.0 – 2.0×10^{-2} M in 10 mM phosphate buffer, pH 7.4) were shaken at 37°C. After equilibrium was attained (5 days), the solution was centrifuged at 2000 g for 5 min and the supernatant was filtered through a membrane filter (Advantec Dismic 13CP, Toyo-Roshi, Tokyo, Japan) and analyzed using HPLC under the same condition described above. The apparent 1:1 stability constant (K_c) of the complexes was calculated using Eq. (1) using the slope and an intercept of the initial straight-line portion of the phase solubility diagrams (Higuchi and Connors, 1965). The 1:1 stoichiometry of β -CyD complexes with benzoic acid derivatives employed in this study was assumed because they showed a straight line in the initial portion of the phase solubility diagrams, as described later.

$$K_c = \text{slope} / \{\text{intercept} (1 - \text{slope})\} \quad (1)$$

3. Theory

Fig. 1 shows a schematic representation for permeation of a drug through a cellophane membrane in the absence

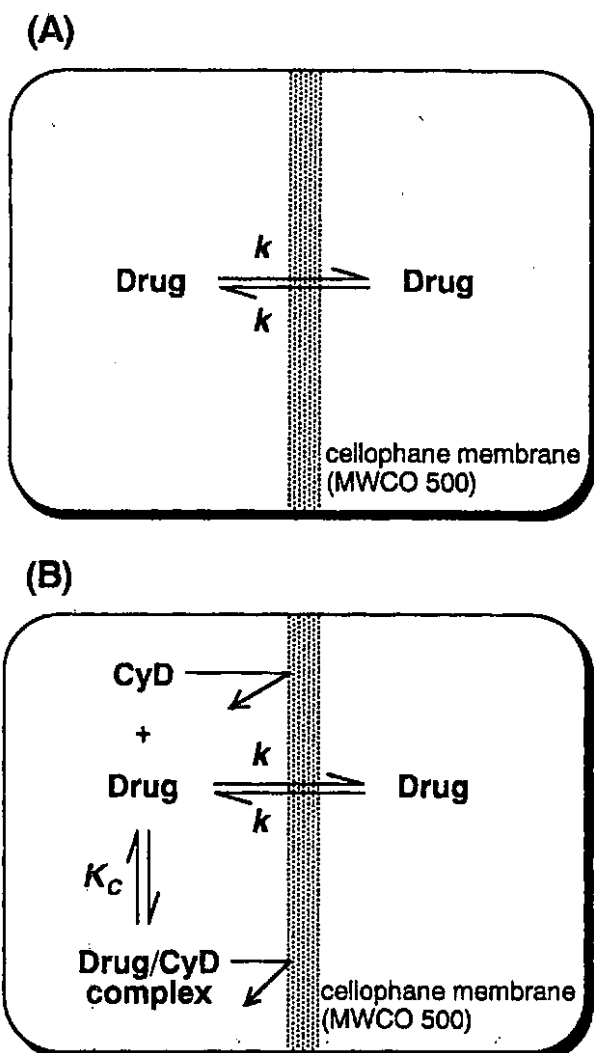


Fig. 1. Permeation of drug in the absence (A) and presence (B) of CyDs in donor phase through a cellophane membrane. k , permeation rate constant of drug; K_c , stability constant of drug/CyD complex.

and presence of CyDs in a donor phase. CyDs and its complexes are assumed to impermeate, because the membrane (Spectra/Por® MWCO 500) employed is permeable only for molecules with molecular weight less than about 500. The permeation rate of a drug in the absence of CyDs is expressed by the well-known Eq. (2) (Lucck et al., 1957).

$$\begin{aligned}
 [D]_t - [D]_{eq} &= ([D]_0 - [D]_{eq}) \exp(-2kt), \\
 &\text{for the donor phase;} \\
 [D]_{eq} - [D]_t &= [D]_{eq} \exp(-2kt), \\
 &\text{for the acceptor phase}
 \end{aligned} \quad (2)$$

where k is the permeation rate constant of the drug and $[D]_0$, $[D]_t$ and $[D]_{eq}$ are concentrations in the donor or acceptor phase at times 0 and t and at the equilibrium,

respectively. In the presence of CyDs in the donor phase, the rate equation for permeation is expressed by Eq. (3):

$$d[D]_A/dt = k[D]_F - k[D]_A \quad (3)$$

where $[D]_F$ and $[D]_A$ are the concentration of free drug in the donor phase and that of a drug in the acceptor phase, respectively. The stability constant (K_c) of the 1:1 complex is defined by Eq. (4):

$$\begin{aligned}
 K_c &= [\text{complex}]/([D]_F[\text{CyD}]_F) \\
 &= ([D]_0 - [D]_F - [D]_A)/([D]_F[\text{CyD}]_F)
 \end{aligned} \quad (4)$$

where $[\text{complex}]$ and $[\text{CyD}]_F$ are concentrations of the complex and the free CyDs in the donor phase, respectively. Eq. (5) is derived from Eq. (4) and substituting Eq. (5) for $[D]_F$ in Eq. (3) yields Eq. (6) for the permeation rate.

$$[D]_F = ([D]_0 - [D]_A)/(1 + K_c[\text{CyD}]_F) \quad (5)$$

$$\begin{aligned}
 d[D]_A/dt &= k\{[D]_0 - (2 + K_c[\text{CyD}]_F)[D]_A\}/ \\
 &\quad (1 + K_c[\text{CyD}]_F)
 \end{aligned} \quad (6)$$

Integrating Eq. (6) yields Eq. (7) (logarithmic form) or Eq. (8) (exponential form), by assuming $[\text{CyD}]_F = [\text{CyD}]_T = \text{constant}$ in the case of the total CyD concentration $[\text{CyD}]_T \gg [D]_0$ (100:1 molar ratio in this study).

$$\begin{aligned}
 \ln\{[D]_0 - (2 + K_c[\text{CyD}]_T)[D]_A\} &= -k\{(2 + K_c[\text{CyD}]_T)/ \\
 &\quad (1 + K_c[\text{CyD}]_T)\}t + \ln[D]_0
 \end{aligned} \quad (7)$$

$$\begin{aligned}
 [D]_A &= [D]_0\{1 - \exp(-At)\}/(2 + K_c[\text{CyD}]_T), \\
 &\text{where } A = k(2 + K_c[\text{CyD}]_T)/(1 + K_c[\text{CyD}]_T)
 \end{aligned} \quad (8)$$

Therefore, the K_c and k values can be determined by analyzing the data of concentrations, $[D]_A$, of a drug in the acceptor phase as a function of time, t , according to Eq. (7) or Eq. (8), using a nonlinear least-squares method of the MULTI program (Yamaoka and Nakagawa, 1983). When the K_c and k values are already known, the plot of the left-hand side of Eq. (7) vs. t gives a straight line.

4. Results and discussion

4.1. Determination of stability constant using the membrane permeation technique

Fig. 2 shows the permeation profiles of phenacetin (1.0×10^{-4} M) in the absence and presence of β -CyD (1.0×10^{-2} M) in the donor phase. Without CyDs, the concentration of phenacetin in the donor phase decreased with time, while that of the acceptor phase simultaneously increased, and both phases were equilibrated at a half of the initial drug concentration after about 60 h. This permeation profile was analyzed according to Eq. (2) and the permeation rate constant (k) of $4.42 \times 10^{-2} \text{ h}^{-1}$ was obtained. There was no dependence of the rate constant on

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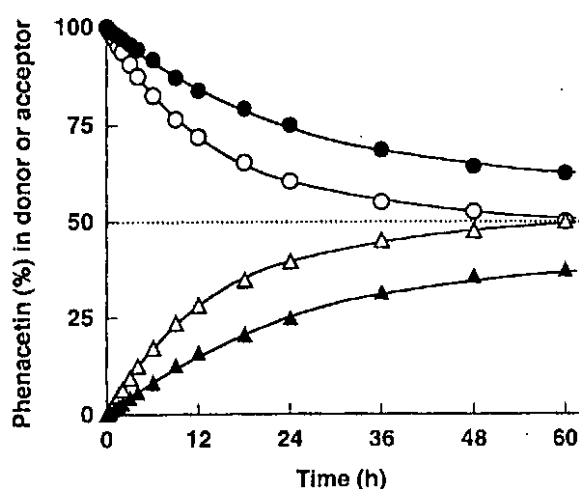


Fig. 2. Permeation profiles of phenacetin (1.0×10^{-4} M) in the absence (open symbols) and presence (closed symbols) of β -CyD (1.0×10^{-3} M) in donor phase through a cellophane membrane (MWCO 500) in isotonic phosphate buffer (pH 7.4) at 37°C . $\circ$, $\bullet$, phenacetin in donor; Δ , $\blacktriangle$, phenacetin in acceptor. The solid lines were theoretical curves drawn using Eqs. (2) and (8).

initial concentrations in the range 1.0×10^{-4} – 1.0×10^{-2} M. The k values for other benzoic acids are listed in Table 1. In the presence of β -CyD, on the other hand, the equilibrium concentration of the drug in the donor phase was higher than that in the acceptor phase. This is due to the complexation of phenacetin with β -CyD in the donor phase, i.e. the total concentration of the drug in the donor phase is a sum of the free and complexed fractions, and only the free fraction of the drug is in equilibrium with that in the acceptor phase (see Fig. 1). Thus, the stability constant (K_c) of the β -CyD complex with phenacetin was calculated using the drug concentrations in the donor phase (6.26×10^{-3} M, 63% of the initial concentration) and the acceptor phase (3.73×10^{-3} M, 37% of the initial concentration) at 60 h and was 171 M^{-1} (S.D. = ± 2 , $n=3$). This method is essentially the same as the equilibrium

dialysis method for the determination of K_c values (Martin et al., 1983). However, this method was time-consuming and it took more than 60 h to attain the equilibrium between the donor and acceptor phases. Therefore, the permeation profile of $[D]_A$ vs. t was analyzed using Eq. (8) and data of an early stage of the permeation (within 6 h) and the unknown parameters, k and K_c values, were determined by a nonlinear least-squares method (Yamaoka and Nakagawa, 1983). The K_c value of the phenacetin- β -CyD complex was 172 M^{-1} (S.D. = ± 3 , $n=3$) and was in close agreement with that determined using the solubility method (182 M^{-1} , S.D. = ± 3 , $n=3$) as described later. The permeation rate constant (k) of phenacetin was $4.63 \times 10^{-2} \text{ h}^{-1}$ which was also in close agreement with that ($4.42 \times 10^{-2} \text{ h}^{-1}$) determined using Eq. (2) and the permeation data in the absence of β -CyD. The plot according to Eq. (7) using the above k and K_c values gave a straight line (slope = -0.063 , intercept = -9.208 , correlation coefficient = 0.999), warranting the above treatment. The K_c value of the phenacetin- β -CyD complex was determined using a conventional kinetic method (Bender and Komiyama, 1978), i.e. the apparent permeation rate constants (k_{obs}) at 0.0 – 1.0×10^{-2} M β -CyD concentrations were calculated using Eq. (2) and the β -CyD concentration dependence of k_{obs} values was analyzed using the Guttman equation (Eq. 9 of Guttman, 1962) assuming the formation of a non-productive complex:

$$k_0/k_{\text{obs}} = K_c[\text{CyD}]_T + 1 \quad (9)$$

where k_0 and k_{obs} are the permeation rate constants of phenacetin (1.0×10^{-4} M) in the absence and presence of β -CyD, respectively. The results were as follows: $k_0 = 4.42 \times 10^{-2} \text{ h}^{-1}$, $k_{\text{obs}} = 3.02 \times 10^{-2} \text{ h}^{-1}$, $2.48 \times 10^{-2} \text{ h}^{-1}$, $1.95 \times 10^{-2} \text{ h}^{-1}$ and $1.61 \times 10^{-2} \text{ h}^{-1}$ in the absence and presence of β -CyD (2.5×10^{-3} M, 5.0×10^{-3} M, 7.5×10^{-3} M and 1.0×10^{-2} M), respectively. The k_{obs} value decreased with the increase in β -CyD concentrations and the plot of k_0/k_{obs} vs. $[\text{CyD}]_T$ according to Eq. (9) gave a

Table 1

Stability constants (K_c) of β -CyD complexes with phenacetin and benzoic acids determined by the solubility method and by the membrane permeation technique and permeation rate constant (k) of guest molecules in isotonic phosphate buffer (pH 7.4) at 37°C

Guest	K_c^a (M^{-1})	K_c^b (M^{-1})	k^c (h^{-1})	k^d (h^{-1})
Phenacetin	182 ± 3	172 ± 0	0.0442	0.0463
<i>o</i> -Toluic acid (<i>o</i> -TA)	109 ± 3	84 ± 0	0.00139	0.00150
<i>m</i> -Toluic acid (<i>m</i> -TA)	159 ± 0	139 ± 0	0.00249	0.00261
<i>p</i> -Toluic acid (<i>p</i> -TA)	214 ± 15	200 ± 2	0.00377	0.00387
<i>o</i> -Chlorobenzoic acid (<i>o</i> -CBA)	164 ± 12	181 ± 3	0.00189	0.00187
<i>p</i> -Chlorobenzoic acid (<i>p</i> -CBA)	36 ± 0	22 ± 1	0.00266	0.00293
<i>m</i> -Bromobenzoic acid (<i>m</i> -BBA)	312 ± 5	267 ± 7	0.00469	0.00483
<i>p</i> -Bromobenzoic acid (<i>p</i> -BBA)	26 ± 0	18 ± 0	0.00279	0.00295
<i>o</i> -Iodobenzoic acid (<i>o</i> -IBA)	283 ± 15	267 ± 9	0.00201	0.00218
<i>m</i> -Iodobenzoic acid (<i>m</i> -IBA)	154 ± 8	150 ± 4	0.00336	0.00354

Each value represents the mean $\pm$ S.D. of three experiments.

^a Stability constants determined by using Eq. (1); and ^b Eq. (8).

^c Permeability rate constants (S.D. $< \pm 0.00002$) of guest molecules determined by using Eq. (2); and ^d Eq. (8).

straight line with a slope of 172 M^{-1} (K_c , S.D. = ± 2 , $n=3$) and an intercept of 0.993. This K_c value coincided with that determined using the membrane permeation technique.

In order to validate the membrane permeation technique, the K_c values of β -CyD complexes with various benzoic acids were determined and compared with those determined using the solubility method. Fig. 3 shows permeation profiles of benzoic acids in the absence and presence of β -CyD in the donor phase. These profiles are analyzed according to Eq. (2) (for the systems without β -CyD) and Eq. (8) (for the systems with β -CyD) using a nonlinear least-squares method. The results of K_c and k values are summarized in Table 1, together with K_c values determined from the phase solubility diagrams of Fig. 4 (see Section 2). The K_c values determined using the permeation technique were in close agreement with those determined using the solubility method, and the experimental data conformed closely to the theoretical permeation curves calculated by Eq. (8). As shown in Fig. 5, the plot of the former K_c values vs. the latter K_c values gave a straight line with a slope of 1.0, an intercept of -11.8 and a correlation coefficient of 0.993. When the data of *o*-TA, *p*-CBA and *p*-BBA were eliminated from the correlation, the plot gave a straight line with a slope of 0.959, an intercept of 1.230 and a correlation coefficient of 0.981, suggesting that the K values determined using the solubility method are slightly higher than those determined by the membrane permeation method, particularly for the complexes having smaller K_c values (<100). This subtle discrepancy may be due to the difference in the experimental conditions, such as concentration range of the host and guest molecules. These results indicate that the membrane permeation technique described here is useful for the determination of the stability constant of CyD complexes.

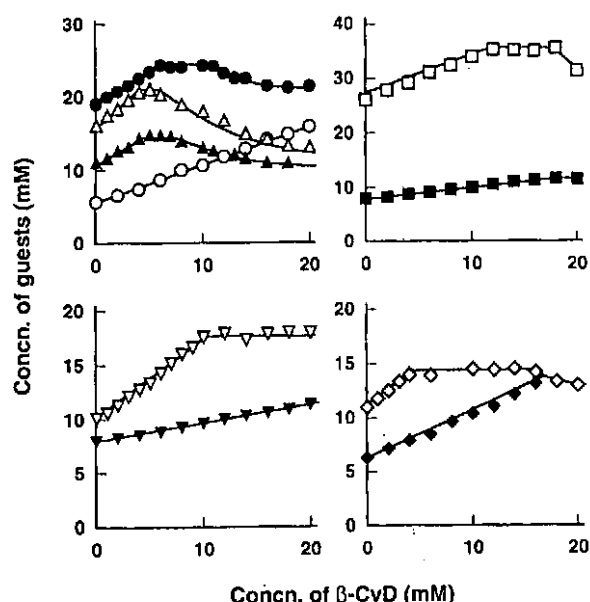


Fig. 4. Phase solubility diagrams of benzoic acid/ β -CyD systems in isotonic phosphate buffer (pH 7.4) at 37°C . $\circ$, phenacetin; $\bullet$, *o*-TA; Δ , *m*-TA; $\blacktriangle$, *p*-TA; $\square$, *o*-CBA; $\blacksquare$, *p*-CBA; ∇ , *m*-BBA; $\blacktriangledown$, *p*-BBA; $\diamond$, *o*-IBA; $\blacklozenge$, *m*-IBA.

4.2. Permeation behavior from drug–competing agent– β -CyD ternary system

The permeation rate of CyD complexes is significantly affected by the presence of second guest molecules, due to competitive inclusion. For example, some part of the complexed fraction dissociates into free drug and CyD due to the competition, thus increasing the fraction of permeable free drug. The degree of the dissociation depends on the relative magnitude of stability constants of the com-

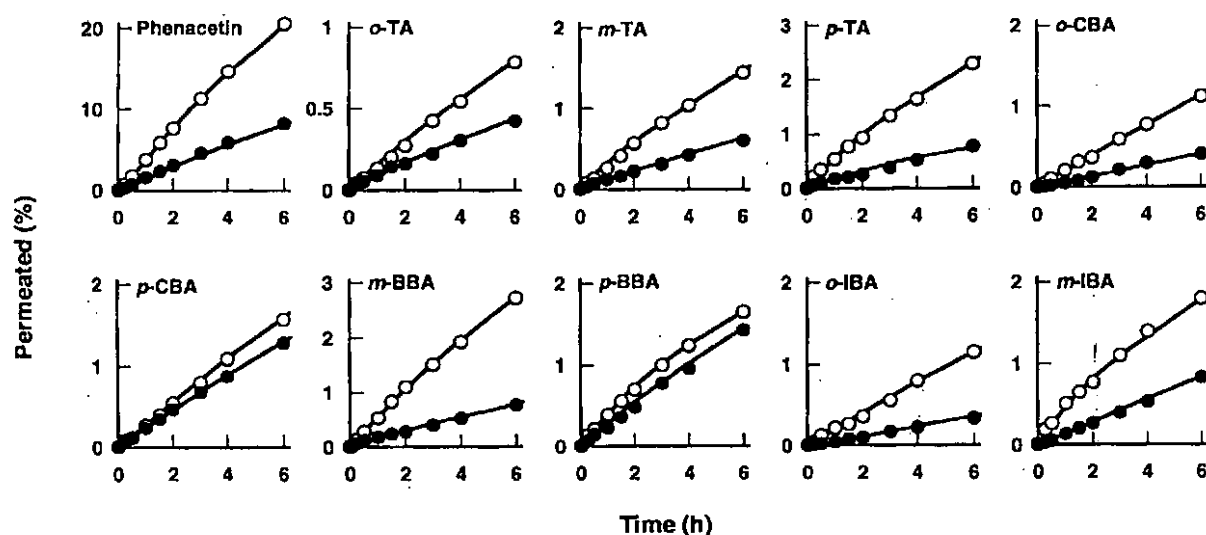


Fig. 3. Permeation profiles of benzoic acids ($1.0 \times 10^{-4} \text{ M}$) in the absence ($\circ$) and presence ($\bullet$) of β -CyD ($1.0 \times 10^{-2} \text{ M}$) through a cellophane membrane (MWCO 500) in isotonic phosphate buffer (pH 7.4) at 37°C . The solid lines were theoretical ones drawn using Eqs. (2) and (8).

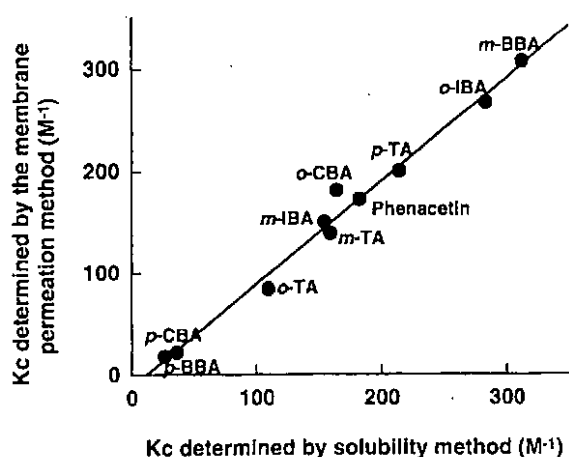


Fig. 5. Plot of K_c values determined by the membrane permeation technique vs. those determined by the solubility method (see Table 1).

plexes with a drug and a competing agent. Such competition may occur in gastrointestinal fluids after oral administration of CyD complexes because of the presence of biological components. Therefore, the membrane permeation behavior of a drug in the presence of competing agents was investigated to justify the validity of the membrane permeation technique for determination of stability constants of CyD complexes and to gain an insight into the permeation of drug–competing agent– β -CyD ternary systems. Fig. 6 shows the permeation profiles of phenacetin (1.0×10^{-4} M) in the absence and presence of *m*-bromobenzoic acid (*m*-BBA, employed as a competing agent, 0.0 – 1.0×10^{-2} M) and β -CyD (1.0×10^{-2} M) in the donor phase. The permeation rate of phenacetin was decelerated by the addition of β -CyD whereas it was

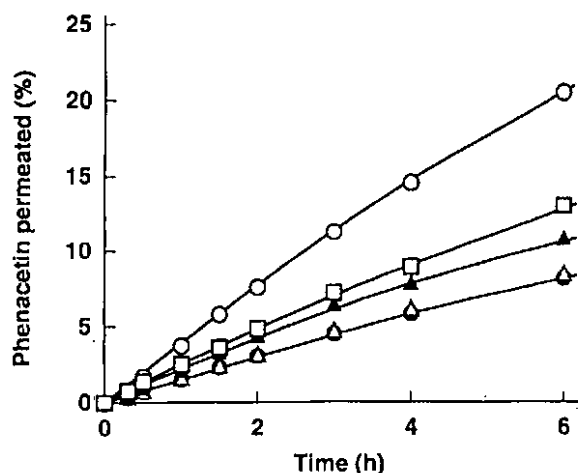


Fig. 6. Permeation profiles of phenacetin (1.0×10^{-4} M) in the β -CyD binary system and the β -CyD/*m*-BBA ternary systems through a cellophane membrane (MWCO 500) in isotonic phosphate buffer (pH 7.4) at 37°C . $\circ$, phenacetin alone; $\bullet$, phenacetin/*m*-BBA/ β -CyD (molar ratio)=1/0/100; Δ , 1/10/100; $\blacktriangle$, 1/50/100; $\square$, 1/100/100. The solid lines were theoretical curves calculated by Runge-Kutta-Gill method using Eqs. (6) and (10).

recovered by the addition of *m*-BBA. *m*-BBA exhibited an insignificant effect on the permeation rate of phenacetin, indicating no interaction between the guest molecule and the competing agent. The permeation of the ternary system is schematically illustrated in Fig. 7, and the permeation rate equations of the drug and the competing agent are described by Eqs. (6) and (10), respectively:

$$\frac{d[\text{CA}]_A}{dt} = k\{[\text{CA}]_0 - (2 + K_{\text{CA}}[\text{CyD}]_F)[\text{CA}]_A\} / (1 + K_{\text{CA}}[\text{CyD}]_F) \quad (10)$$

where $[\text{CA}]_0$ and $[\text{CA}]_A$ are the concentrations of the competing agent at times 0 and t , respectively, and K_{CA} is the stability constant of the β -CyD complex with the competing agent. In contrast to the drug– β -CyD binary system, $[\text{CyD}]_F$ in Eq. (10) can not be approximated as $[\text{CyD}]_F = [\text{CyD}]_T = \text{constant}$, because the concentration of β -CyD was only slightly higher than that of *m*-BBA (molar ratio of *m*-BBA/ β -CyD=1/10–1/1). Therefore, the differential equations Eqs. (6) and (10) were numerically integrated using the Runge-Kutta-Gill method (Yamaoka and Nakagawa, 1983), in order to simulate the permeation profiles. The $[\text{CyD}]_F$ values were estimated using the values of K_c , K_{CA} , $[\text{CyD}]_T$, $[D]_0$ and $[\text{CA}]_0$. As shown in Fig. 6, the permeation data of phenacetin in the ternary system fitted closely to the theoretical curves, supporting the permeation mechanism depicted in Fig. 7.

In conclusion, the stability constant of β -CyD complexes with phenacetin and benzoic acids were determined using the membrane permeation technique using a cellophane membrane permeable for guests but impermeable to β -CyD. This technique was validated as useful for the determination of the stability constant, particularly in the case of 1:1 stoichiometry, and it is more advantageous than the conventional methods, because in the membrane permeation technique the stability constant can be obtained

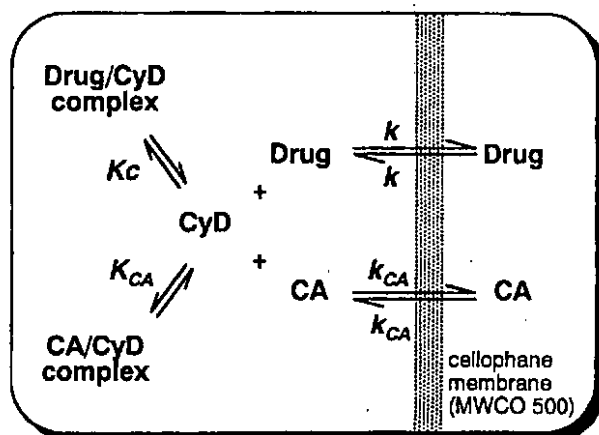


Fig. 7. Permeation of drug in the β -CyD/competing agent ternary system. k , permeation rate constant of drug; k_{CA} , permeation rate constant of competing agent; K_c , stability constant of the complex with drug; K_{CA} , stability constant of the complex with competing agent.

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from only one experimental run by analyzing the permeation data as a function of time. The permeation profiles of phenacetin in the drug-competing agent- β -CyD ternary systems fitted closely to those of the theoretical curves calculated using the stability constants. Knowledge of this sort may be useful for the simulation of absorption behavior of drugs in gastrointestinal tracts after oral administration of CyD complexes, although it can not directly be applied since the appearance and disappearance of biological competing agents and their concentrations are not fully known.

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