Facultad de Farmacia

Departamento de Farmacia Y Tecnología Farmacéutica

VNiVERSiDAD D SALAMANCA

ESTUDIO DE LA PENETRACIÓN DE AGENTES ANTIINFECCIOSOS EN MACRÓFAGOS UTILIZANDO ERITROCITOS PORTADORES

TESIS DOCTORAL

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2009

VNiVERSiDAD
D SALAMANCA

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En calidad de directores de la Tesis cuyo título es "Estudio de la penetración de agente antiinfecciosos en macrófagos utilizando eritrocitos portadores" realizada por la Licenciada en Farmacia Dª ELSA BRIONES CUESTA, consideran finalizado el trabajo y autorizan su presentación a fin de que pueda ser juzgada por el Tribunal correspondiente.

Y para que así conste, firman la presente en Salamanca, a 15 de Julio de 2009.

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AGRADECIMIENTOS

Quiero expresar mi agradecimiento a los Doctores José Martínez Lanao y Clara Isabel Colino Gandarillas, por la dirección de esta tesis, por su confianza, y por su dedicación para sacar este trabajo adelante.

A todos los miembros del Departamento de Farmacia y Tecnología Farmacéutica, por su apoyo, de una u otra manera, durante todos estos años. A Julián y Toñi, por estar siempre dispuestos a echar una mano.

A mis compañeros del laboratorio, a los que siguen aquí, María José, Glenda, Pepe, a los que ya se han ido, Salvador, Nora, Bea, etc., y a todos los que han pasado por el departamento durante estos años, por las horas de trabajo y de agobios compartidas, pero sobre todo, por vuestra amistad y comprensión, por vuestros consejos, por los cafés, las cañas, las risas…

Thanks a lot to the people in the Child Health Department of the St. George´s Hospital of London, especially to Dr. Bridget Bax, for her hospitality and support during my stay there. Many thanks to all the people I met during that time, Juan, Nevio, I am really pleased to meet you.

Por supuesto, quiero dar las gracias a toda la gente que ha vivido esta etapa de mi vida desde "el otro lado", por preocuparos por mí y por aguantarme cuando no me aguantaba ni yo. A todos mis amigos, a los de Burgos, a los de Salamanca, con los que paso mucho tiempo y con los que sólo hablo de vez en cuando, por estar siempre ahí, por hacerme ver las cosas de otra manera.

Y muy especialmente a mi familia, a mis padres y hermanos, que les ha tocado sufrir a veces los "daños colaterales" de la tesis, por apoyarme y por animarme siempre, por confiar en mí. A mis sobrinos, por hacerme reír tanto con cada tontería nueva que aprenden.

Por si me olvido de alguien, quiero dar las gracias a toda la gente que ha pasado por mi vida, para bien o para mal, durante estos años, porque de todo se aprende, y, gracias a todos, además del Doctorado me llevo un "Master" en muchas cosas que no tienen nada que ver con la farmacocinética.

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Introducción y Objetivos

La primera barrera defensiva del organismo frente a agentes infecciosos está constituida por las células fagocíticas, que son capaces de reconocer, ingerir y destruir al microorganismo causante de la infección (Ernst y Stendahl 2006). Existen distintos tipos de células fagocíticas como los leucocitos polimorfonucleares (neutrófilos o granulocitos), que son capaces de migrar desde la sangre al lugar de la infección en respuesta a estímulos quimiotácticos, y los monocitos, presentes en el torrente circulatorio, que van a penetrar en los diferentes tejidos dando lugar a los macrófagos residentes.

La fagocitosis consiste en la ingestión del microorganismo por la célula y su posterior destrucción y eliminación. El fagocito reconoce al microorganismo por medio de receptores de superficie y lo internaliza formando una vesícula o fagosoma, que se funde con los lisosomas dando lugar al fagolisosoma, donde el microorganismo va a ser degradado por los enzimas lisosomales. Además, estas células producen una serie de agentes bactericidas que contribuyen a esta acción, como derivados de oxígeno o iones halogenados en el caso de los neutrófilos, y proteínas catiónicas y óxido nítrico en los macrófagos (Reinoso et al. 1995, Hazenbos y Brown 2006).

A pesar de esto, algunos microorganismos son capaces de crecer y reproducirse una vez que han sido ingeridos por las células fagocíticas, bien evitando la fusión de fagosoma y el lisosoma, o resistiendo la acción de los enzimas en el fagolisosoma. En estos casos el germen sobrevive en uno u otro compartimento celular, o en el citoplasma si son capaces de romper o atravesar la membrana del fagolisosoma (Reinoso et al. 1995, Ahsan et al. 2002). La localización intracelular de estos microorganismos les protege frente a los mecanismos de defensa del organismo, como los anticuerpos o el complemento, o frente la acción de antibióticos que no son capaces de penetrar en las células (Holmes et al. 1966, Carryn et al. 2003). Esto explica la dificultad en el tratamiento de infecciones causadas por parásitos intracelulares obligados, y la importancia patológica de los reservorios intracelulares en infecciones causadas por microorganismos facultativos,

que pueden evitar la acción de los antibióticos penetrando en las células y abandonar la célula huésped cuando la concentración de antibiótico es lo suficientemente baja. En ocasiones esto puede provocar recaídas, o bien derivar en enfermedades crónicas (Klempner 1984, Carryn et al. 2003).

Debido a su mayor capacidad fagocítica, sus propiedades bactericidas menos potentes, y su larga semivida, monocitos y macrófagos son los reservorios celulares más habituales (Maurin y Raoult 2001).

Existen numerosos microorganismos capaces de provocar infecciones intracelulares. Entre ellos se encuentran bacterias como *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Brucella*, *Listeria*, *Legionella pneumophila*, *Salmonella*, *Chlamydia psittaci*; virus como *VIH*, *Influenza*, *Herpes simplex*; hongos como *Aspergillus fumigatus* o *Candida albicans*; o protozoos como *Leismania* (Briones et al. 2008).

Algunos antibióticos como las quinolonas, los macrólidos o la clindamicina tienen la capacidad de penetrar en las células por distintos mecanismos, pero existen muchos agentes antiinfecciosos, como los antibióticos betalactámicos y aminoglucósidos, o algunos antirretrovirales, que no consiguen atravesar la membrana celular (Hand et al. 1987). Estos últimos tienden a permanecer en el espacio extracelular, no llegando a alcanzar concentraciones terapéuticas en el interior de la célula donde se encuentra el patógeno. Esto supone que muchos de estos agentes, con eficacia probada en estas infecciones *in vitro*, muestren escasa o nula actividad *in vivo* (Prokesch y Hand 1982).

La concentración intracelular del agente antiinfeccioso está condicionada por diversos aspectos como el balance entre la entrada y salida del fármaco, y otros procesos como el metabolismo, la unión a distintos componentes celulares o la acumulación en compartimentos subcelulares como el fagolisosoma. Además, su actividad intracelular está influenciada por otros factores como el estado metabólico del microorganismo, las características físico-químicas del lugar de infección, o el grado de cooperación con las defensas del hospedador (Carryn et al. 2003, Van

Bambeke et al. 2006, Barcia-Macay et al. 2006). Desde un punto de vista farmacodinámico la concentración del fármaco es crítica en antibióticos como los macrólidos, fluoroquinolonas y aminoglucósidos, y el tiempo de exposición es importante en betalactámicos y glicopéptidos (Maurin y Raoult 1994, Kutlin et al. 1999, Carryn et al. 2003).

Por otro lado, la amplia distribución del antibiótico o antirretroviral por todo el organismo y su presencia en tejidos no infectados, además de su inherente toxicidad, pueden llevar a la aparición de efectos indeseados que hacen su uso inapropiado, a pesar de mostrar una buena actividad (Lutwyche et al. 1998, Adams et al. 1999, Katragadda et al. 2000, Sinha et al. 2000, Schiffelers et al. 2001).

Una estrategia para mejorar la penetración celular consiste en incorporar el fármaco en sistemas portadores que van a ser ingeridos por las células fagocíticas, dirigiendo éste de forma selectiva a las células diana (Briones et al. 2008, Buxton 2009, Debbage 2009).

Estos sistemas portadores deben ser biodegradables y biocompatibles, y mantenerse estables en las condiciones *in vivo* en las que van a ser utilizados (Roser et al. 1998). En comparación con la administración del fármaco en su forma libre, presentan una serie de ventajas que se resumen a continuación (Katragadda et al. 2000, Schiffelers et al. 2001, Torchilin 2006, Basu y Lala 2006, Schiffelers et al. 2006, Briones et al. 2008, Misra 2009):

- Permiten solucionar problemas tecnológicos de solubilidad, estabilidad, etc.
- Una vez en el torrente circulatorio son reconocidos y retirados de la circulación por las células fagocíticas del sistema retículo-endotelial (SRE) donde reside el patógeno, por lo que se van a alcanzar elevadas concentraciones en las células diana.
- Proporcionan una liberación sostenida del fármaco, manteniendo niveles terapéuticos en el lugar de infección durante largos periodos de tiempo.

- Evitan la acumulación del fármaco en tejidos que no pertenecen al SRE, disminuyendo la aparición de efectos adversos.
- Evitan la degradación prematura del fármaco por la acción de enzimas plasmáticas y la aparición de reacciones inmunológicas.
- Aumentan la retención del fármaco en los tejidos.
- Minimizan la aparición de resistencias por los bajos niveles de permeación en las bacterias y células fagocíticas.
- Permiten aumentar el intervalo terapéutico del fármaco al disminuir su toxicidad manteniendo su eficacia terapéutica.

La vectorización puede ser activa o pasiva. La vectorización pasiva se produce por la capacidad inherente de las células fagocíticas para ingerir a los sistemas portadores, a los que reconoce como sustancias extrañas al organismo. Este fenómeno está favorecido por la opsonización de los portadores por las proteínas séricas cuando llegan al torrente circulatorio. La vectorización activa se basa en modificaciones en la superficie de los portadores que aumentan su afinidad por las células diana. Esto se consigue mediante la unión de ligandos que van a ser reconocidos específicamente por algunos receptores de las células fagocíticas, facilitando su internalización mediante endocitosis mediada por receptores (Mukhopadhyay y Basu 2003, Vasir et al. 2005). Otra alternativa es la fijación de anticuerpos específicos del agente causante de la infección, lo que aumenta la selectividad por las células infectadas.

Existen distintos sistemas portadores de agentes antiinfecciosos, entre los que están liposomas, micro- y nanopartículas poliméricas, nanosuspensiones, o conjugados con lipoproteínas, y portadores de naturaleza biológica como los eritrocitos, leucocitos polimorfonucleares o "ghost" bacterianos.

Los eritrocitos son las células más abundantes del organismo. Su principal función es el transporte de oxígeno de los pulmones al resto de los tejidos. Tienen una vida útil de 120 días. Cuando los eritrocitos envejecen son retirados de la

circulación por los macrófagos y eliminados, especialmente, por el hígado y el bazo (SRE) (Gothoskar 2004, Gutiérrez et al. 2004a). Debido a su ubicuidad y su relativa simplicidad interna, han sido considerados como una buena alternativa para la vehiculización de fármacos en el organismo (Lanao y Sayalero 2006).

Los eritrocitos han sido ampliamente estudiados como potenciales vectores de fármacos y macromoléculas por sus distintas aplicaciones en el campo de la medicina, ya que pueden actuar como reservorios del fármaco proporcionando una liberación sostenida del mismo durante largos periodos de tiempo, o dirigir el fármaco de manera selectiva a órganos del SRE, especialmente hígado, bazo y médula ósea. Esta última aplicación es de especial importancia en el caso de los agentes antiinfecciosos para el tratamiento de infecciones intracelulares, ya que su incorporación en eritrocitos portadores consigue aumentar considerablemente la penetración en las células fagocíticas donde reside el patógeno (Briones et al. 2008).

Las ventajas derivadas de la utilización de los eritrocitos como sistemas portadores son (Bax et al. 1999, Magnani et al. 2002, Hamidi y Tajerzadeh 2003, Gutiérrez et al. 2004a, Gothoskar 2004, Lanao y Sayalero 2006, Hamidi et al. 2007b, Pierigè et al. 2008, Patel et al. 2008, Serafini et al. 2009):

- Presentan una elevada biocompatibilidad, especialmente cuando se utilizan eritrocitos autólogos, y biodegradabilidad, sin generar productos tóxicos.
- Son fáciles de obtener del paciente y fáciles de manipular.
- Debido a la ausencia de núcleo y otros orgánulos tienen un elevado volumen acuoso, lo que permite una elevada eficacia de encapsulación. Esto garantiza la obtención de una dosis suficiente con un volumen pequeño de células.
- No requieren un transportador específico para la encapsulación, lo que aumenta el espectro de fármacos que pueden ser incorporados.
- Tienen sistemas enzimáticos por lo que pueden actuar como biorreactores, transformando un profármaco encapsulado en su forma activa.

- Muestran un aclaramiento sistémico similar al de los eritrocitos no tratados, por lo que tienen una vida útil en circulación mayor que otro tipo de sistemas portadores. Esto permite una liberación sostenida del fármaco durante largos periodos de tiempo.
- Son retirados de la circulación por las células fagocíticas del SRE, lo que proporciona una mayor selectividad en estos tejidos. Además existen procedimientos que permiten aumentar la fagocitosis de los eritrocitos portadores, aumentando la selectividad.

Sin embargo, el uso de eritrocitos como sistemas portadores presenta también algunos inconvenientes (Álvarez et al. 1995, Jain y Jain 1997, Moss et al. 2000, Valbonesi et al. 2001, Sugai et al. 2001, Gutiérrez et al. 2004a):

- La rápida eliminación de los eritrocitos por los órganos del SRE puede producir problemas toxicológicos.
- Algunas sustancias difunden rápidamente a través de la membrana del eritrocito impidiendo su llegada al SRE.
- Muestran una gran variabilidad y difícil estandarización debido a su origen biológico.
- Presentan problemas de almacenamiento.
- Requieren rigurosos controles en su obtención y manipulación para evitar problemas de contaminación biológica.

Los primeros intentos de encapsular sustancias en eritrocitos se realizaron en los años 50, con adenosin trifosfato (ATP) (Gardos 1953) y dextrano (Marsden y Ostling 1953). Sin embargo, la primera vez que se utilizó este tipo de sistemas portadores con una aplicación farmacológica fue en 1973, cuando se encapsuló β-galactosidasa y β-glucosidasa en eritrocitos humanos para su utilización en reemplazamiento enzimático (Ilher et al. 1973). En 1979 se introdujo el término

"carrier erythrocytes" para describir a los eritrocitos portadores de fármacos (Jain y Jain 1997, Gothoskar 2004). Desde entonces, el uso de eritrocitos como sistemas portadores de fármacos y otras sustancias se ha extendido a numerosos campos de la medicina, como son el tratamiento de infecciones intracelulares (DeLoach y Wagner 1984, DeLoach 1985, Berman y Gallalee 1985, Mishra et al. 1996, Rossi et al. 2004a, Gutiérrez et al. 2005, 2008a,b, Murphy et al. 2006), del SIDA (Magnani et al. 1992, 1995, Benatti et al. 1996, Fraternale et al. 2002, Serafini et al. 2009), en reemplazamiento enzimático (Beutler et al. 1977, Adriaenssens et al. 1984, Bax et al. 2000, 2007), en tratamientos antineoplásicos (Zocchi et al. 1989, Mishra y Jain 2002, Lotero et al. 2003), enfermedades inflamatorias (Ogiso et al. 1985, Rossi et al. 2001, 2004b, Annese et al. 2005, 2006, Lucidi et al. 2006, Castro et al. 2006, 2007), eliminación de agentes tóxicos (Muthuvel et al. 2006), etc. Más recientemente se están empleando para la encapsulación de material genético para su aplicación en terapia génica (Magnani et al. 2002, Larson et al. 2004, Byun et al. 2004, Hamidi et al. 2007b), anticuerpos para el desarrollo de vacunas (Murray et al. 2006, Hamidi et al 2007a), interferón (Hamidi et al. 2007c), agentes de contraste para resonancia magnética (Brähler et al. 2006) y en inmunosupresión (Rossi et al. 2008).

Sin embargo, la mayoría de los estudios realizados con eritrocitos portadores están en fase preclínica, ya que no existe un método adecuado de almacenamiento que permita su elaboración y conservación a gran escala, lo que limita la generalización de su uso clínico. A pesar de ello, se han realizado con éxito algunos ensayos clínicos como la encapsulación de glucocerebrosidasa para el tratamiento de la enfermedad de Gaucher (Beutler et al. 1977), adenosina deaminasa para tratar la deficiencia de este enzima (Bax et al. 2000, Bax et al. 2007), asparraginasa en leucemia linfoblástica (Kravtzoff et al. 1996), o corticoesteroides para el tratamiento de la enfermedad pulmonar obstructiva crónica (Rossi et al. 2001), fibrosis quística (Rossi et al. 2004b, Lucidi et al. 2006) o enfermedad inflamatoria intestinal (Annese et al. 2005, 2006, Castro et al. 2006, 2007). En todos los casos,

los tratamientos demostraron una buena eficacia sin aparición de efectos adversos, y una excelente tolerancia y aceptación por los pacientes. Actualmente, la unidad de terapia celular del Hospital Debrousse de la Universidad de Lyon, asociada a la empresa francesa ERYtech Pharma dispone de la tecnología patentada Erycaps® que permite la encapsulación de enzimas, fármacos y otras sustancias terapéuticas para su utilización clínica.

Existen diferentes métodos para la encapsulación de sustancias en eritrocitos, que pueden ser físicos (electroporación) (Haritou et al. 1988, Mangal et al. 1991, Lizano et al. 1998, 2001), osmóticos (dilución hipotónica, prehinchamiento hipotónico, diálisis hipotónica) (Gutiérrez et al. 2004a,b, Gothoskar 2004, Pierigè et al. 2008), o químicos (perturbación química de la membrana del eritrocito) (Matovcik et al. 1985, Tonetti et al. 1991). Los métodos osmóticos son los más utilizados. Se basan en el comportamiento de los eritrocitos en respuesta a los cambios de tonicidad del medio, de manera que en contacto con un medio hipotónico se hinchan y en un medio hipertónico se contraen.

Entre los métodos osmóticos el más frecuentemente utilizado es el método de diálisis hipotónica, ya que es el que mejor preserva las características bioquímicas y fisiológicas de los eritrocitos, y permite conseguir una eficacia de encapsulación relativamente alta. Para llevarlo a cabo, una suspensión de eritrocitos con el fármaco a encapsular se deposita en el interior de una membrana de diálisis que se sumerge en el medio hipotónico, de manera que los eritrocitos aumentan su volumen hasta que se forman poros en la membrana celular que van a permitir la entrada del fármaco. A continuación la membrana de diálisis se transfiere a un medio hipertónico donde los eritrocitos se contraen y recuperan su forma bicóncava. Los poros de la membrana desaparecen quedando el fármaco retenido en el interior del eritrocito (Gothoskar 2004, Gutiérrez et al. 2004b, Pierigè et al. 2008).

Dada la importancia de los reservorios intracelulares en el tratamiento de ciertas infecciones, y la potencial utilidad de los sistemas portadores para su tratamiento, en este trabajo se ha estudiado la utilización de eritrocitos de rata

como sistemas portadores de diferentes agentes antiinfecciosos. Para ello se han seleccionado dos agentes antiinfecciosos como fármacos modelo: un antibiótico, amicacina, de naturaleza hidrófila, y un antirretroviral, zidovudina (AZT), de naturaleza lipófila.

La amicacina es un antibiótico aminoglucósido, de origen semisintético, que es efectivo frente a infecciones causadas por bacterias aeróbicas Gram (-) y actúa sinérgicamente frente a algunas Gram (+). Su mecanismo de acción se basa en la inhibición de la síntesis proteica al unirse al ARN ribosómico, destruyendo la integridad de la membrana celular (González y Spencer 1998, Jana y Deb 2006, Shakil et al. 2008). Su actividad es similar a la de otros aminoglucósidos, pero con la ventaja de una mayor resistencia a la inactivación enzimática, por lo que es activa frente a bacterias resistentes a gentamicina y tobramicina, y presenta un mejor perfil farmacocinético (Ristuccia y Cunha 1982, Ristuccia y Cunha 1985, Mensa et al. 2003).

Sin embargo, a pesar de su demostrada eficacia, su utilidad clínica se ve limitada por los graves efectos adversos que provoca, especialmente nefro- y ototoxicidad. Por otro lado, debido a su carácter policatiónico presenta una elevada solubilidad en agua lo que impide su difusión a través de membranas celulares. Así su localización va a ser fundamentalmente extracelular, de manera que su eficacia frente a infecciones provocadas por bacterias intracelulares va a ser limitada (Maurin y Raoult 2001)

El VIH1 infecta a los macrófagos y linfocitos T-helper (CD4+), pero la principal característica de la enfermedad del SIDA es la depleción de las células CD4+. Sin embargo, el sistema monocito-macrófago, especialmente los macrófagos residentes en los tejidos, son capaces de resistir el efecto citopático del virus, de manera que van a actuar como reservorios a largo plazo permitiendo la distribución del virus en todos los tejidos. Así el virus se localiza en compartimentos como el bazo, los pulmones, la médula ósea o el sistema nervioso central, algunos de ellos difícilmente accesibles por la mayoría de los fármacos antirretrovirales disponibles.

Éstos no pueden alcanzar concentraciones terapéuticas durante un tiempo suficiente en el lugar de infección, dando lugar a la aparición de efectos adversos debido a las elevadas dosis necesarias, y al desarrollo de resistencias. La consecuencia es el fracaso del tratamiento y la progresión de la enfermedad (Aquaro et al. 2002, Perno et al. 2006, Govender et al. 2008, Ojewole et al. 2008, Serafini et al. 2009).

La zidovudina es un antirretroviral del grupo de los inhibidores de la transcriptasa análogos de nucleósidos, y es uno de los más utilizados en el tratamiento del SIDA. Tras su administración es absorbido rápidamente alcanzando la concentración máxima en plasma aproximadamente en 1 hora. Tiene una biodisponibilidad del 65% debido a que sufre efecto de primer paso, y su semivida tiene un valor de 1 hora. Esto hace que para mantener niveles terapéuticos sea necesaria la administración frecuente de grandes dosis (200 mg cada 4 horas). Esta dosificación hace que se alcancen niveles tóxicos produciendo efectos adversos graves como granulocitopenia y anemia (Garg y Jain 2006).

Tanto en el caso de los aminoglucósidos como en el de los antirretrovirales la utilización de sistemas portadores va a permitir una acumulación más selectiva del fármaco en tejidos del SRE donde se localiza el patógeno, mejorando así la eficacia del tratamiento, y evitando la aparición de efectos adversos debidos a la presencia del fármaco en otros órganos y tejidos no infectados (Hu et al. 2000, Lanao et al. 2007, Briones et al. 2008).

El primer objetivo de este trabajo ha sido realizar una completa revisión bibliográfica acerca de la problemática en el tratamiento de las infecciones intracelulares. También se recogen los distintos tipos de sistemas portadores existentes en la actualidad y las ventajas que éstos aportan en el tratamiento de estas infecciones, ya que facilitan el acceso de los agentes antiinfeciosos al interior celular.

El segundo objetivo de este trabajo ha sido la puesta a punto y validación de distintas técnicas analíticas que permitan la cuantificación de los fármacos estudiados en diferentes tipos de muestras biológicas, como eritrocitos, macrófagos,

plasma y tejidos. Se han puesto a punto una técnica de HPLC-fluorescencia para la cuantificación de amicacina, una técnica de HPLC-ultravioleta para la cuantificación de zidovudina en eritrocitos, y una técnica de UPLC-MS/MS para la cuantificación de zidovudina en plasma y tejidos.

El tercer objetivo de este trabajo ha sido la puesta a punto y optimización de métodos que permitan la encapsulación en eritrocitos de rata de los fármacos seleccionados. Para la encapsulación de amicacina se ha utilizado una técnica de diálisis hipotónica que ha sido puesta a punto previamente en nuestro laboratorio (Gutiérrez et al. 2005). Posteriormente, esta técnica se ha optimizado para la encapsulación del antirretroviral zidovudina. Para ello se ha estudiado la influencia de diferentes factores como la cantidad inicial de fármaco, el tiempo de diálisis, y la relación de volúmenes diálisis/tampón hipotónico, tanto en el rendimiento de encapsulación como en las propiedades hematológicas de los eritrocitos.

Finalmente, el cuarto objetivo de este trabajo ha sido el estudio de la penetración de amicacina y zidovudina en macrófagos tanto *in vitro* como *in vivo*, así como los cambios en la farmacocinética y en la distribución tisular de estos fármacos en ratas cuando se administran incorporados en eritrocitos portadores por vía intraperitoneal, en comparación con su utilización en forma libre.

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CAPÍTULO 2

Delivery systems to increase the selectivity of antibiotics in phagocytic cells

Journal of Controlled Release (2008) 125, 210-27

Delivery systems to increase the selectivity of antibiotics in phagocytic cells.

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Abstract

Many infectious diseases are caused by facultative organisms that are able to survive in phagocytic cells. The intracellular location of these microorganisms protects them from the host defence systems and from some antibiotics with poor penetration into phagocytic cells. One strategy used to improve the penetration of antibiotics into phagocytic cells is the use of carrier systems that deliver these drugs directly to the target cell. Delivery systems such as liposomes, micro/nanoparticles, lipid systems, conjugates, and biological carriers such as erythrocyte ghosts may contribute to increasing the therapeutic efficacy of antibiotics and antifungal agents in the treatment of infections caused by intracellular microorganisms. The main objective of this review is to analyze recent advances and current perspectives in the use of antibiotic delivery systems in the treatment of intracellular infections such as mycobacterial infections, brucellosis, salmonellosis, lysteriosis, fungal infections, visceral leishmaniasis, and HIV.

Keywords: delivery systems; antibiotics; phagocytic cells; intracellular infections; selectivity

1. Introduction

Phagocytic cells are an essential component of the immune system and their main function is to ingest and destroy microorganisms [1]. There are different types of phagocytic cells, such as blood polymorphonuclear leucocytes, (neutrophils or granulocytes), able to migrate to sites of infection. Monocytes are another type of phagocytic cell that are also found in the blood stream. When monocytes leave the circulation and penetrate tissues, they change shape and become macrophages.

Pathogenic microorganisms are sometimes able to survive and reproduce after they have been ingested by phagocytic cells, especially macrophages, which hinders the treatment of this type of infection. The intracellular location of these microorganisms protects them from the host defence mechanisms and from the action of antibiotics, which may encounter difficulties in penetrating phagocytic cells [2, 3].

Pathologies as widespread as leishmaniasis, tuberculosis or histoplasmosis, among others, are caused by intracellular microorganisms. They are also the cause of opportunistic infections in immunodepressed patients, especially those with AIDS, where infections due to mycobacteria involve more complications [4].

The antibiotics mainly used today in the treatment of this type of infections belong to different groups, such as the aminoglycosides, the fluoroquinolones, the beta-lactams, the macrolides, etc. These drugs are characterized by having different ability to penetrate in phagocytic cells, which may limit their efficacy in the treatment of intracellular infections. The use of antibiotic delivery systems with capacity for selective distribution in phagocytic cells is an important resource in improving antibiotic therapy against intracellular infections.

The main objective of this review is to analyse the different non-biological and biological delivery systems used to improve the uptake of antibiotics by phagocytic cells, as well as the *in vitro* and *in vivo* studies performed with these types of carrier and their efficacy against different types of pathogens.

2. Therapeutic interest of the uptake of antibiotics by phagocytic cells.

Phagocytosis consists of the ingestion of microorganisms by the cell and their later destruction and elimination. The process is similar for macrophages and neutrophils. The phagocyte uptakes the microorganism by means of surface receptors, a process sometimes mediated by antibodies, and internalises it, forming a vesicle or phagosome, which then fuses with lysosomes to give rise to a phagolysosome. These cells produce also a series of bactericidal agents that contribute to this action, such as derivatives of oxygen, halogen ions in the case of neutrophils, and cationic proteins and nitric oxide in the case of macrophages [5, 6] (Figure 1).

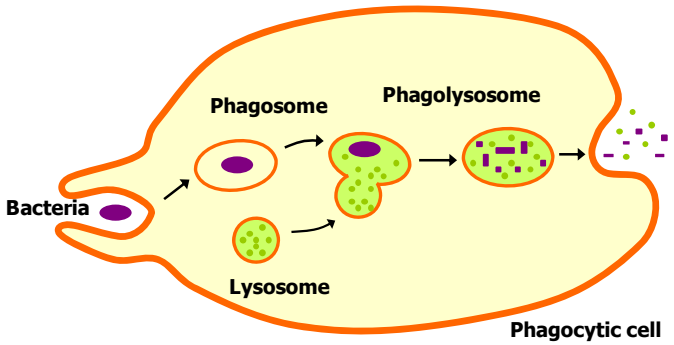

Figure 1. Phagocytosis of microorganisms by phagocytic cells.

When phagocytic cells are unable to destroy the germs responsible for an infection, either by preventing fusion of the phagosome and lysosome or by resisting the microbiocidal mechanisms in the phagolysosome, the germs survive in one cellular compartment or another, or in the cytoplasm if they are able to break down or cross the membrane [5, 7].

The intracellular location of these microorganisms protects them from the host defence mechanisms, such as antibodies or complement, and from the action of antibiotics that are unable to penetrate the cell [2, 3]. This explains the difficulty involved in treating infections caused by obligate intracellular microorganisms and

the pathological importance of intracellular reservoirs in infections caused by facultative microorganisms, since they can prevent the action of the antibiotic by penetrating in cells and leaving the host cell when the level of antibiotic is low enough, causing further relapses [3, 8]. Moreover, these reservoirs may lead to chronic disease. Owing to their greater phagocytic capacity, less potent microbicidal properties and their long half-lives, monocytes and macrophages are the usual reservoirs [9]. Consequently, the intraphagocytic accumulation of antibiotics is of great therapeutic interest for combating this type of infection.

Phagocytic cells are also able to transport drugs from the blood or a tissue to the site of infection by chemotactic mechanisms and, once there, release the drug. Accordingly, the capacity of an antibiotic to become concentrated within phagocytic cells is also important in its action against extracellular microorganisms located at the site of infection or inflammation [10-12].

Intracellular infections can be produced by different bacteria, such as *Mycobacterium tuberculosis, M. Leprae, Brucella, Listeria, Legionella pneumophila, Salmonella, Chlamydia psittaci*, viruses such as *HIV, influenza, herpes simplex,* fungi such as *Aspergillus fumigatus, Candida albicans*, or parasites such as *Leishmania* (Table 1).

Type of infection	***Microorganism***	***Disease***	***Antibiotic***
Gram (-) Bacterial	*Brucella species*	Brucellosis	Aminoglycosides, tetracyclines, amphenicols, lincosamides, polymixins, quinolones, sulphamides, trimetroprim
	Escherichia coli	Diarrheal illness	
	Legionella pneumophila	Pneumonia	
	Pseudomonas aeruginosa	Pneumonia, endocarditis, bacteremia, meningitis	
	Salmonella species	Salmonellosis, typhoid fever	
	Shigella dysenteriae	Bacillary dysentery	
	Rickettsiae	Typhus, Rocky Mountain Spotted Fever	
	Yersinia pestis	Plague	
Gram (+) Bacterial	*Listeria monocytogenes*	Listeriosis, meningitis, septicemia	Betalactams, tetracyclines, amphenicols, macrolides, lincosamides, vancomycin, bacitracin, 3rd generation quinolones, sulphamides, trimetroprim
	Mycobacterium avium complex	Opportunistic infections in inmmunocompromised patients	
	Mycobacterium leprae	Leprosy	
	M. tuberculosis	Tuberculosis	
	Staphylococcus aureus	Pneumonia, mastitis, phlebitis, meningitis, urinary tract infections, osteomyelitis, endocarditis, nosocomial infections	
Protozoarian	*Leishmania donovani*	Visceral leishmaniasis	Pentavalent antimonials, polyene antibiotics, pentamidine, sulphamides, pyrimethamine, suramine, melarsoprol, nifurtimox, tryparsamide
	Leishmania infantum	Visceral leishmaniasis	
	Leishmania major	Cutaneous leishmaniasis	
	Toxoplasma gondii	Toxoplasmosis	
	Trypanosoma cruzi	Chagas disease	
Fungal	*Aspergillus fumigatus*	Aspergillosis	Polyene antibiotics, imidazolics, flucytosine, terbinafine
	Candida albicans	Candidiasis	
	Cryptococcus neoformans	Cryptococcosis	
	Histoplasma capsulatum	Histoplasmosis	
Viral	*Herpes simplex* type 1 and 2	Oral and genital herpes	Interferons, monoclonal antibodies, neuraminidase inhibitors, nucleosides, nucleotides. Antiretrovirals
	HIV	AIDS	

Table 1. Main infections produced by intracellular microorganisms and the antibiotics usually employed for their treatment.

3. Penetration of phagocytic cells by antibiotics.

Once administered to the organism, many currently used antibiotics tend to remain in the extracellular space. The treatment of intracellular infections is a serious problem for classic antimicrobial therapies since it requires therapeutic antibiotic concentrations to be reached inside the cells. This means that many antibiotics with proven efficacy against intracellularly located pathogens *in vitro* show little or no antibacterial activity *in vivo*, considering that the antibiotic must be present at a sufficiently high concentration to be able to destroy the microorganism directly or to increase the antibacterial function of phagocytic cells [13].

Most antimicrobials have a limited capacity to penetrate cells (□-lactams and aminoglycosides) and only a few (quinolones, macrolides and clindamycin) are efficiently taken up by phagocytic cells [14]. There are several mechanisms by which antibiotics penetrate cells, as shown in figure 2. Cell penetration may occur through diffusion, as in the case of the fluoroquinolones, beta-lactams and macrolides, or by receptor-mediated uptake, as in the case of aminoglycosides [3].

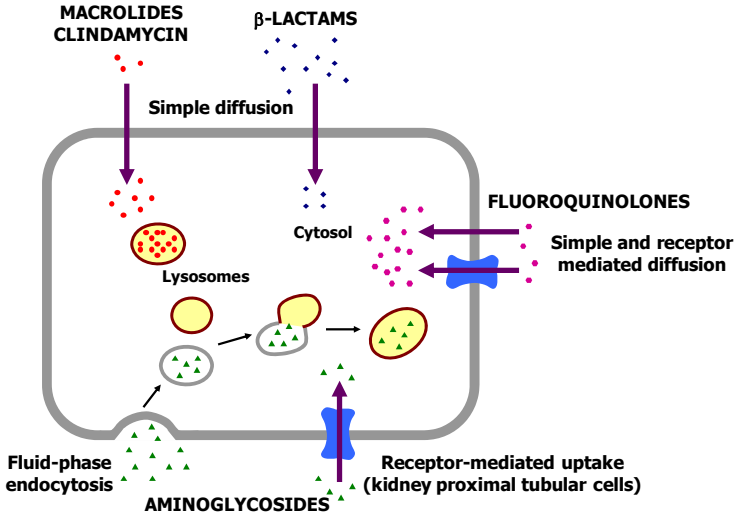

Figure 2. Mechanisms by which antibiotics penetrate phagocytic cells.

The intracellular concentration of the antibiotic depends on the balance between input and output, drug metabolism and binding or accumulation in different intracellular structures like phagolysosomes. The cell accumulation of the antibiotic is not always predictive of the intracellular activity. Antibacterial activity of the intracellular antibiotic is influenced by the state of bacterial responsiveness, physicochemical environment at the site of infection and the degree of cooperation with the host defenses [3, 15, 16]. It also depends on the pathogen being in a metabolic state that is sensitive to the action of the antibiotic, and on the influence of the drug on the phagocytic capacity of the host cell, since sometimes it may activate it while on other occasions it inhibits such activity [3, 16-18] (Figure 3).

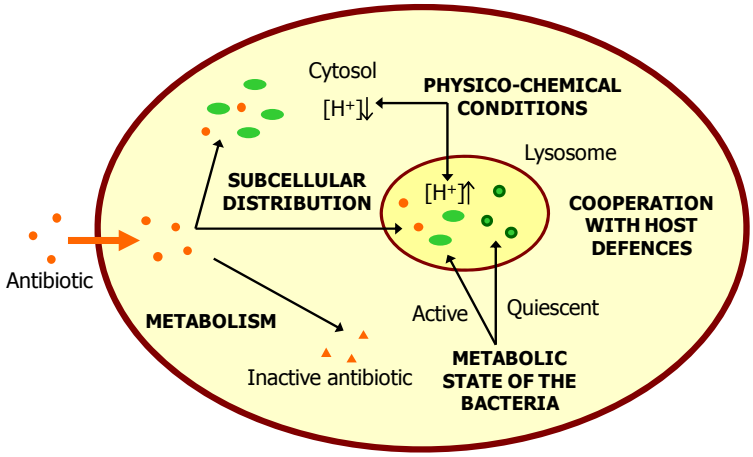

Figure 3. Factors affecting the intracellular activity of antibiotics.

From a pharmacodynamic point of view intracellular concentration of the antibiotic is critical in drugs like macrolides, fluoroquinolones and aminoglycosides and the time of exposure is important in beta-lactams and glycopeptides. Intracellular environment like acid pH of lysosomes and related vacuoles may decrease the activity of aminoglycosides or macrolides [3, 19, 20]. Evaluation of intracellular activity of different antibiotics using an *in vitro* model of infected THP-1 human macrophages with *Staphylococcus aureus* demonstrate that the intracellular activities are lower than that observed extracellularly and dependent on the

extracellular concentration and the time lasting duration of cell exposure to antibiotics [16].

Although entry into the cell is a necessity, not all antibiotics that penetrate phagocytic cells will have a therapeutic effect [13]. This is the case of clindamycin, which is strongly concentrated in cells but has low or no activity against sensitive microorganisms, possibly because it inhibits the antimicrobial action of phagocytic cells [12, 21-24].

Other factors, such as the difficulty involved in penetrating the cell at doses lower than the maximum one tolerated, the scarce retention of drugs that rapidly diffuse back out of the cell, low stability in the intracellular environment or degradation by lysosomal enzymes render antibiotics inefficacious against intracellular microorganisms. Also, the broad distribution of these drugs throughout the organism and their presence in uninfected tissues, apart from their inherent toxicity, may lead to undesirable effects that make their use inappropriate, even though their antimicrobial activity is good [25-29].

One strategy used to improve penetration consists of incorporating the antibiotic into delivery systems that are later ingested by phagocytic cells, selectively delivering the antibiotic to the target cell. Moreover, the ingestion of such vehicles may involve macrophage activation, increasing the immune response of the host [30].

4. Delivery systems in antibiotic therapy for phagocytic cells.

Carrier systems allow antibiotics to be delivered selectively to phagocytic cells and to increase their cellular penetration in order to treat intracellular infections, especially in the case of antibiotics active against microorganisms that produce this type of infection but that have a low intracellular penetration capacity.

These carrier systems must be biodegradable and biocompatible, and they must remain stable under the *in vivo* conditions in which they are to be used [31].

Additionally, they should have a series of specific characteristics, summarized as follows [4, 26, 27, 29, 32-38]:

1. The ability to solve the technical problems of solubility, stability, etc., when certain active principles are formulated.
2. Once in the bloodstream, they must be rapidly recognised and withdrawn from the circulation by the phagocytic cells of the reticuloendothelial system (RES), where the pathogen is located, to reach elevated drug concentrations in the target cells.
3. They should allow sustained release of the drug, to achieve therapeutic levels at the site of infection over long periods of time.
4. They must prevent the drug from manifesting its pharmacological and toxicological actions until the RES has been reached, hence decreasing the incidence of side effects.
5. The ability to prevent premature degradation of the encapsulated drug and prevent immunological reactions.
6. Drug retention in tissues must be increased.
7. They must minimise the appearance of resistances due to low drug permeation levels in bacteria and phagocytic cells.
8. They should be able to increase the therapeutic index of the drug, decreasing its toxicity and maintaining its therapeutic efficacy.

Vectoring may be passive or active. Passive vectoring is enabled by the inherent capacity of phagocytic cells to ingest carrier systems, which are recognized as substances foreign to the organism. This phenomenon is favoured by the opsonisation of carriers by serum proteins when they arrive in the bloodstream. Active vectoring uses surface modifications in the carriers, which then develop an affinity for recognizing and specifically interacting with target cells. This is accomplished by the binding of ligands, which are recognised by specific receptors

of phagocytic cells, to which they bind with great affinity, thereby facilitating their internalisation via receptor-mediated endocytosis. Mannosyl/fucosyl receptors and macrophage scavenger receptors are those most studied in this sense. A common practice is to use glycoproteins or polysaccharides ending in mannose or fucose radicals, and polyanionic macromolecules such as acetylated LDL lipoproteins, with affinity for scavenger receptors [39, 40]. Another alternative is the fixation of specific antibodies of the agent responsible for the infection, such that the selectivity for infected cells is increased.

Release profiles of the drug from nanoparticles depends on the nature of the delivery system. The drug is released from the liposomes into the biophase both by passive diffusion of the drug through the bilayer as well as when the liposome is degraded in the lysosome [32]. Synthetic polymers carrier systems can release the drug by: polymer degradation or chemical cleavage of the drug from the polymer, swelling of the polymers and releasing the drug entrapped within them, osmotic pressure effects creating pores and releasing drugs by simple diffusion mechanisms [37, 41].

Antibiotic carrier systems vary in nature. Examples are liposomes, micro- and nanoparticles, nanosuspensions and conjugates with water-soluble polymers and with lipoproteins. Other carrier systems are of a biological nature, such as cell ghosts.

4.1. Liposomes.

Liposomes may be defined as microscopic vesicles made up of one or two phospholipid bilayers surrounding an aqueous compartment. Those with a single phospholipid bilayer are known as single-layer vesicles (SLV) and the others as multilayer vesicles (MLV). They are usually composed of phospholipids, natural or synthetic, and cholesterol, and they may incorporate other lipids and derivatives, and proteins. Their particle size is in the range of 25 nm to several microns, such that they can be used to encapsulate hydrophilic drugs in the aqueous phase, or hydrophobic drugs bound to or incorporated in the lipid bilayer. They are

biodegradable, have low toxicity and immunogenicity, and a high drug/vehicle ratio [42, 43].

Among their drawbacks are their low stability in the bloodstream and during storage, the low encapsulation efficiency, especially toward hydrophobic drugs, and the presence of residues of toxic solvents in the final preparation. Regarding large-scale production, the main problem is the costliness of their manufacture [30, 44].

Despite lipids used in liposome production show relatively low toxicity, several studies have shown liposome toxicity to different types of cells. Positively charged liposomes showed toxicity to bucal cells *in vitro*, while negatively charged liposomes toxicity was relatively low, being dipalmitoylphosphatidylcholine (DPPC) the best choice as main lipid in both positively and negatively charged liposomes [45]. Human skin fibroblasts were sensitive to unilamelar liposomes of phospatidylcoline (PC), soya-PC being more toxic than dimyristoyl-PC [46]. Stearylamine-, cardiolipin-, phosphatidylglycerol-, and phosphatidylserine-containing liposomes were toxic to different cultured human cell lines, and showed that, in general, small liposomes were more toxic than large ones [47]. Liposomes formulated with cationic lipids were toxic to phagocytic macrophages and monocyte-like cells, but not to non-phagocytic T lymphocytes [48]. Experimental animals showed symptoms of neurotoxicity when they were treated with different liposomal formulations [49, 50]. So, when preparing liposomes is important the choice of main lipid, the charge component and the amount of charge component to obtain liposomes with minimum toxicity [45].

When liposomes are administered *in vivo*, they are rapidly removed from the circulation by monocytes and macrophages and are accumulated in the organs of the RES, especially the liver and spleen. This tendency of liposomes is an advantage in the treatment of intracellular infections involving this type of cell. Additionally, the monocyte/macrophage system plays an important role in non-specific defence against infections and the phagocytosis of liposomes can activate it, improving non-specific resistance to certain infections [42].

The main mechanism by which liposomes are captured by phagocytic cells follows several steps: stable adsorption onto the cell membrane, vesicle internalization through an energy-dependent mechanism, fusion of the endocytic vesicles with the lysosomes, and degradation of the liposomes by lysosomal enzymes, releasing the drug encapsulated within them [7, 51] (Figure 4).

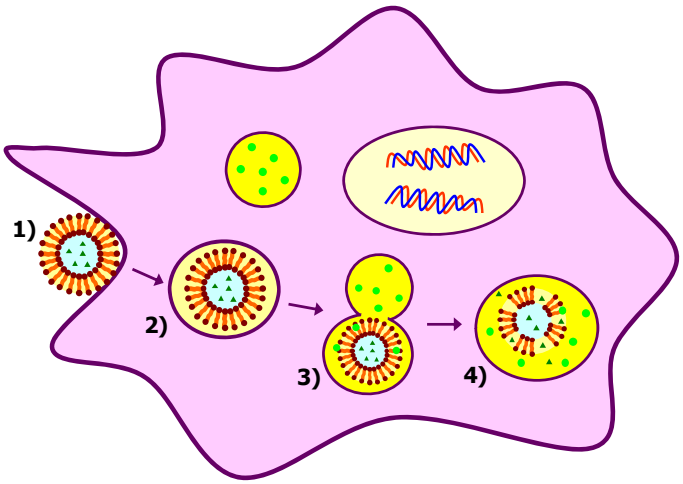

Figure 4. Main mechanisms of penetration of liposomes in phagocytic cells: 1) adsorption onto the cell membrane; 2) vesicle internalization; 3) fusion of the vesicle with the lysosome; 4) degradation of the liposome and drug release.

This is useful in the treatment of infections due to germs residing in the lysosome but not when they are in the cytoplasm. In the latter case, pH-sensitive liposomes are used, these including components that in the endosome will destabilise the lipid bilayer due to the decreased pH, thus favouring fusion of the liposome with the endosomal membrane and release of the drug into the cytoplasm [25, 32, 52].

The limiting step in these processes is the binding of the liposome to the cell surface, such that obtaining stable adsorption is crucial for favouring the penetration of the carrier into the cell. Other possible mechanisms of interaction are enzymatic degradation of the lipid bilayer, with the consequent release of drug in

the vicinity of the cell; the exchange of lipids with the cell, and non-facilitated diffusion through the cell membrane, which is of special importance for hydrophobic drugs [27].

In order for liposomes to be able to bear biologically active substances to phagocytic cells, certain conditions must be met. The liposomes must bind to and be phagocytosed by macrophages (free and tissue-bound); they must prevent the degradation of the encapsulated drug; they must maintain the drug encapsulated until they arrive at the cells of the reticuloendothelial system, and they must direct themselves to the organs where the disturbances associated with macrophages are occurring [7].

The size, composition, cholesterol content and surface properties of the lipid bilayer determine the degree of penetration of liposomes in macrophages [7, 27, 29, 42].

Capture can be modified by incorporating ligands that favour or inhibit the interaction with macrophages in order to increase drug entry into the cell or to prolong the half-life of the liposomes in plasma. The use of the tetrapeptide tuftsin, an activator of phagocytic cells, incorporated into liposomes increases the selectivity for phagocytic cells such as monocytes, macrophages and polymorphonuclear leucocytes [53].

The existence of negative charges favours the binding of serum proteins to the lipid surface, in turn favouring and increasing resistance to serum HDL and hence stability in the bloodstream. Negatively charged liposomes containing phosphatidylserine and dicetylphosphate were capable of delivering greater amount of drug into the macrophages than either neutral or positive liposomes [27]. Moreover, positively charged liposomes show better monocyte activation. There are also other mechanisms of liposome internalization, such as fusion or endocytosis, due to the electrostatic attraction between the positive charges of the liposome and the negative charges of the cell surface [25, 54].

The inclusion of cholesterol or sphingomyelin in the composition of liposomes, which is a frequent practice, increases the rigidity of the lipid bilayer, which then hinders interaction with the cell. Accordingly, the presence of increasing amounts of cholesterol will decrease the cellular penetration of vesicles [7, 27]. Cholesterol also favours the stability of liposomes against serum lipoproteins by increasing the time of circulation in the blood and decreasing the percentage of encapsulation of the drug because it modifies the thickness of the membrane [51, 54].

4.2. Micro/nanoparticles.

These are stable, solid, polymeric particles that can incorporate drugs either inside them or bound to the polymeric matrix [55-58]. They are made of biodegradable polymers, of natural or synthetic origin, that are degraded *in vivo* enzymatically, non-enzymatically or by a combination of both, to give non-toxic products that are readily eliminable by the organism through the usual metabolic routes. The use of natural polymers such as bovine serum albumin (BSA), human serum albumin (HSA), collagen, gelatine and haemoglobin is limited owing to their high cost and debatable purity. Accordingly, it is now more common to use synthetic polymers, such as poly(amides), poly(amino acids), poly(alkyl-α-cyanoacrylates), poly(esters), poly(orthoesters), poly(urethanes) and poly(acrylamides), owing to their high biodegradability and biocompatibility [59].

Their behaviour *in vivo* is similar to that of liposomes, and they are rapidly captured by phagocytic cells and are mainly accumulated in organs such as the liver, spleen and bone marrow [60].

In comparison with liposomes, they have the advantage of greater encapsulation efficiency, greater stability in the presence of serum and during storage and their usefulness for achieving sustained release [61, 62].

Currently there are very few drugs on the market that have been formulated with micro/nanoparticles owing to the relative toxicity of some of the polymers used

and because of the lack of suitable and cost-effective methods for large-scale production of these systems [63, 64].

Nanoparticles can affect biological behaviours at the cellular, subcellular and protein level. Due to its small size, they can be distributed throughout the body, reach target tissues and access to undesiderable sites in the cell, such as the nucleus or mitochondria, causing genetics damage and mutations [64, 65].

Some polymers used as implants are not suitable as nanoparticles, because in the nanometer size polymers are uptaken by cells and degradated inside them, and it can cause cytotoxic effects [63, 64]. Microspheres of poly(lactic acid) (PLA) showed toxicity in mouse peritoneal macrophages [66]. Polylactide/glycolide (PLA/GA) nanoparticles reduced the viability of human granulocytes due to the intracellular degradation of the polymers [67].

Variants of microparticles are lipid micro/nanoparticles. Solid Lipid Nanoparticles™ (SLN) are a hybrid between polymeric microparticles and liposomes. They have the advantages of fatty emulsions (large-scale production, the non-use of organic solvents, and low toxicity) and of polymeric nanoparticles (controlled release due to the solid lipid matrix) [68]. They are made of a solid matrix composed of physiological and well tolerated lipids stabilised in the form of aqueous suspensions by means of a surfactant covering. They are appropriate for the encapsulation of lipophilic drugs. Despite the presence of the surfactants, they have less toxicity than polymeric microparticles. SLN showed to be less toxic than PLA/GA nanoparticles in a culture of human granulocytes [67]. Besides, their degradation is faster, affording better controlled release of the drug at the site of action [63, 69-73]. They also allow oral, parenteral or even pulmonary administration through inhalers.

4.3. Nanosuspensions.

These include particles in the nanometre size range in suspension that contain the drug and the smallest amount of surfactant possible, normally in

aqueous suspension. They are used in the case of drugs that -owing to their low solubility- show problems in absorption and bioavailability. Their formulation as nanosuspensions solves these obstacles, allowing them to be administered orally and parenterally. Additionally, because of their size nanoparticles are readily ingested by phagocytic cells, it thus being possible to raise drug levels in the intercellular compartment [71, 74].

4.4. Conjugates with water-soluble polymers and with lipoproteins (LDL).

The binding of a drug to a polymer allows endocytosis of the conjugate by macrophages by means of specific receptors. This is especially useful for sparingly water-soluble drugs. These conjugates improve the solubility of the drug, the time it remains in the systemic circulation and its accumulation inside cells. Arabinogalactan, HPMA, or dextran are used to make these carrier systems, sometimes with mannose ligands to increase selectivity [75-77].

Dendrimers are synthetic water-soluble polymers with a tree-like branching structure that have found important applications in nanotechnology medicine [78, 79]. Recently, water-soluble dendrimers have been used for the targeting of rifampicin, chloroquine and lamivudine to macrophages [80-82]. However, many studies show that cationic dendrimers show citotoxicity in different cell lines [83, 84]. Dendrimer citotoxicity is dependent on the chemistry of the core, but is mainly influenced by the nature of the dendrimer surface [84].

Low-density plasma lipoproteins (LDLs) are quasi-spherical endogenous nanoparticles with an approximate diameter of 22 nm, formed by a lipid core surrounded by a monolayer of phospholipids in which cholesterol and apoprotein-B (Apo-B) are present [85, 86]. Their metabolism involves internalisation by LDL receptors, present in nearly all the cells of the organism, through the Apo-B lipoprotein present in the LDLs and in cells of the monocyte-macrophage system by scavenger receptors [57, 87, 88]. Chemical modifications typical of the metabolism of Apo-B such as glycosylation, acetylation, aldehyde derivatization, and oxidation affect the affinity of LDLs for the scavenger receptors of phagocytic cells.

Accordingly, the binding of drugs to modified LDLs may be used as a strategy for transporting these substances into the cell interior for the treatment of intracellular infections. Owing to their lipid nature, they are useful for the transport of lipophilic drugs [87]. One limitation of this carrier system is their unstable nature [89]. However, little is known about the influence of the drug incorporation on the structural integrity of the LDL, which affects to metabolic processes as receptor binding or enzyme activity, to asses the utility of these lipoproteins as a drug delivery system [90].

They can be readily obtained from human plasma by density gradient ultracentrifugation. There are several methods for incorporating drugs into LDLs. The incubation time is an important parameter in clinical practice since rapid incorporation will be beneficial in the sense of the possibility of administering the patient's own LDLs once these have been loaded with drug [85].

4.5. Cells and cell ghosts

Drug-containing erythrocytes, known as erythrocyte ghosts, and more recently bacterial ghosts are able to selectively direct antibiotics to the phagocytic cells of the RES, and are potentially useful for the treatment of infections caused by intracellular microorganisms [91].

Erythrocytes account for most of the blood cells and are the main transporters of oxygen to the cells and tissues of the organism. When they age, they are lysed in the circulation or phagocytosed in the RES, especially in the spleen, where they are attacked by lysosomal enzymes, which cause the cell membrane to break and haemoglobin to degrade.

Erythrocytes are potential biocompatible vectors for different bioactive substances, including drugs and proteins. They may find clinical application as drug reservoirs able to provide sustained release in the organism, or as vehicles to selectively direct drugs to the RES, especially the liver, spleen and bone marrow. This latter application is of special relevance in the case of anti-infective agents for

the treatment of intracellular infections because they are able to improve, indeed to a considerable extent the penetration of such drugs into phagocytic cells. The advantages of erythrocytes as antibiotic carrier systems can be summarized as follows [33, 91-102]:

1. High biocompatibility and stability as compared with other carrier systems.
2. They are easy to obtain from the patient by venipuncture and are easy to handle.
3. A high aqueous volume, permitting relatively high efficiency in encapsulation.
4. They do not require a specific transporter for encapsulation, which increases the spectrum of drugs susceptible to being encapsulated.
5. Encapsulation in erythrocytes provides the drug in question with a systemic clearance similar to that of erythrocytes, allowing sustained therapeutic levels of drug to be maintained in the blood for long periods of time and generating a sustained release of drug to the RES.
6. Erythrocytes have enzymatic systems, such that they can act as bioreactors, transforming the encapsulated prodrug into the active principle.
7. It is possible, by different procedures, to increase the phagocytosis of carrier erythrocytes by the RES.

However, the use of erythrocytes as carrier systems also has some disadvantages [101-107]:

1. The rapid elimination of erythrocytes by the RES may sometimes elicit toxicological problems.
2. Some substances diffuse rapidly through the erythrocyte membrane, preventing them from arriving at the RES.
3. They show great variability owing to their biological origin.
4. They have storage problems.

5. There are problems related to biological contamination, such that rigorous controls of their collection and manipulation are required.

Another cell system used as delivery system of antibiotics are the polymorphonuclear leukocytes. Human polymorphonuclear leukocytes has been used as potential carriers of different antibiotics mainly macrolides and azalides like cethromycin, dirithromycin and azithromycin [10, 108, 109]. A slow release of antibiotics like azithromycin from loaded PMN has been described [110]. Apoptosis, is a process in which the cells induce their own death in response to different types of stimulus. Apoptosis is reflected in enhanced membrane permeability, which favours the release of the encapsulated drug from the apoptotic cells to the tissue [91]. Apoptopic neutrophils show a selective phagocytosis by macrophages and may be used as a potential delivery system for the targeting of antibiotics to macrophages [111, 112].

More recently, bacterial ghosts have been proposed as delivery systems for drugs and genetic material. Bacterial ghosts are intact, non-denatured bacterial cell envelopes produced by protein E-mediated lysis of Gram-negative bacteria. The specificity of bacterial ghosts for phagocytic cells, together with their capacity for encapsulating water-soluble substances makes them potential candidates for the encapsulation of antibiotics [91, 113, 114].

Table 2 shows the main carrier systems used as antibiotic delivery systems that allow their selective distribution within the RES to be improved.

Carrier system	*Encapsulated drug*	*Reference*
Liposomes	Gentamicin	[25, 52, 54, 115, 116]
	Amikacin	[117]
	Ciprofloxacin	[118-120]
	Ofloxacin	[121]
	Sparfloxacin	[122]
	Rifampicin	[123, 124]
	Isoniazid	[124]
	Clofazimine	[26]
	Resorcinomycin A	[125]
	Azithromycin	[118, 119]
	Clarithromycin	[121, 126]
	Ampicillin	[127]
	Amphotericin B	[53, 128-138]
	Nystatin	[139-147]
	Sodium stibogluconate	[148]
	Pentavalent antimony	[149]
	Pentamidine isethionate	[150]
	Amarogentin	[151]
	Andrographolide	[28]
	Primaquine	[152]
	Stavudine	[27]
	Dideoxycytidine	[153]
Immunoliposomes	Doxorubicin	[154]
Micro/nanoparticles	Gentamicin	[61, 155, 156]
	Ampicillin	[157]
	Rifampicin	[158-160]
	Amphotericin B	[161-164]
	Zidovudine	[56, 165]
	Zalcitabine	[55]
	Saquinavir	[55]
Nanosuspensions	Aphidicolin	[166]
Polymers	NPC1161 (8-aminoquinolone)	[77]
	Norfloxacin	[76]
	Amphotericin B	[75, 167]
Dendrimers	Lamivudine	[80]
	Rifampicin	[81]
	Chloroquine	[82]
Lipoproteins (LDL)	Zidovudine prodrugs	[87]
	Flucitosine	[168]
	Zidovudine	[168]
Erythrocytes	Gentamicin	[98]
	Adefovir	[169]
	bis-PMEA	[170]
	ACVpPMPA	[171]
	Dideoxycytidine	[172, 173]
	AZTp2AZT	[174]
Polymorphonuclear leukocytes	Cethromycin	[108]
	Azythromycin	[10, 110]
	Dirithromycin	[109]

Table 2. Delivery systems for antiinfective drugs.

5. In vitro and *in vivo* antibacterial activity of carrier systems.

Several *in vivo* and *in vitro* studies have reported the potential applications of different carrier systems to increase the selectivity of antibiotics for phagocytic cells and bolster therapeutic efficiency in the treatment of intracellular infections.

5.1 Infections due to mycobacteria.

Infections due to mycobacteria are an important health problem since these microorganisms are responsible for severe diseases such as tuberculosis and leprosy, among others, and are in turn responsible for opportunistic infections in immunodepressed patients, such as individuals with AIDS.

Tuberculosis, caused by *Mycobacterium tuberculosis*, is a common lung infection that is even endemic to certain regions. Its incidence has increased recently because it is often associated with AIDS. The *M. avium-M. intracellulare* (MAC) complex is the main cause of complications in immunodepressed patients [125, 175].

There are drugs that are efficient against tuberculosis, but these are used in prolonged treatment, increasing the risk of side effects [42, 123]. MAC is resistant to most classic anti-tuberculosis drugs owing to the low permeability of cells or rapid degradation, such that new drugs have been developed, although in practice they do not provide very good results [117, 176]. The use of delivery systems facilitates the selective shuttling of antibiotic to the site of infection and such systems provide slow and sustained drug release, which allows administration over longer intervals of time. However, once in the blood, most of the antibiotic administered is captured by liver and spleen cells but not so much by alveolar macrophages, limiting efficiency in lung infections [176].

The encapsulation of different antibiotics in liposomes has shown good antibacterial efficacy in both macrophage cell lines and in animal models of MAC-due disease [122, 177, 178].

The aminoglycoside antibiotics are very efficacious against infections due to mycobacteria, but they are unable to cross cell membranes, such that they cause a whole range of side effects; in particular, nephro- and ototoxicity [52]. The bactericidal activity of gentamicin and amikacin in liposomes is increased in the liver and spleen of mice infected with MAC, but not in lungs. A more prolonged action is obtained, which allows intermittent administration [115, 117].

Clofazimine is the drug of choice for leprosy, although it is also used in cases of tuberculosis resistant to other drugs, and it shows activity against MAC. It does penetrate macrophages, but it is not known whether it reaches effective intracellular concentrations. Its encapsulation in MLVs increases its efficiency in mice infected with MAC, in the liver, spleen, kidneys and, to a lesser extent, in the lung, and decreases its *in vitro* and *in vivo* toxicity, allowing higher doses to be administered. It also allows parenteral administration, which in free form would be impossible owing to problems of solubility [4, 34]. In the treatment of chronic and acute tuberculosis in mice, liposomal clofazimine administered parenterally maintained its antibacterial properties, decreasing toxicity. However, in that study the drug was mainly located in the liver and spleen, so that the use of aerosols would be appropriate in respiratory infections [22].

Clarithromycin is a semisyntheic macrolide antibiotic with anti-mycobacterial activity. It is accumulated in macrophages, but accesses the phagosome, where the bacterium resides, only with difficulty. Different types of liposomes containing clarithromycine increase -to a greater or lesser extent- the cellular penetration of the drug; hence its efficacy against mycobacteria in human macrophages [121, 126]. Azitromycin efficiently inhibits the growth of *M. avium in vitro* using negatively charged liposomes in a macrophage cell line [118] and *in vivo* using specific stealth liposomes in a mouse model of tuberculosis infection [119].

Resorcinomycin A is a new antibiotic that is active against MAC strains that are resistant to other drugs. Its encapsulation in MLVs increases its cellular

penetration and activity in mouse macrophages infected with MAC and affords sustained action over 7 days [125].

Quinolones are accumulated in phagocytic cells but their intracellular efficacy is limited. Their encapsulation in carrier systems may solve formulation problems and afford controlled release [120, 179]. Ciprofloxacin efficiently inhibits the growth of *M. avium in vitro* in a murine macrophage-like cell line using negatively charged liposomes [118] and *in vivo* using specific stealth liposomes in a mouse model of tuberculosis infection [119]. Similar results have been obtained using stealth liposomes of isoniazid and rifampicin, which exhibit controlled release and reduce toxicity *in vivo* in mice infected with *M. tuberculosis* [^{124}]. Ofloxacin loaded liposomes increased the concentration of the drug and reduced the number of colony-forming units (CFUs) in MAC-infected human macrophages [121]. The combination of norfloxacin and a dextran polymer bound to mannose decreases the number of CFUs in the lungs of mice infected with *M. bovis*, whereas in solution it is ineffective. Binding is accomplished through a tetrapeptide by an α(N-C) bond sensitive to lysosomal proteases, allowing release in the lysosomes [76].

Rifampicin is the drug of choice for the treatment of tuberculosis. The appearance of resistance and side effects is common. The incorporation of the peptide tuftsin, able to activate macrophages, on the surface of liposomes containing rifampicin increases the concentration of the drug in the liver, spleen, and lungs of healthy rats [42, 53]. The use of liposomes in the form of aerosols and with ligands allows greater accumulation in rat alveolar macrophages, both *in vitro* and *in vivo*. Owing to its affinity for scavenger receptors of macrophages, MBSA (maleylated bovine serum albumin) has been used as a ligand, because it is an anionic compound, together with O-SAP (O-steroyl-amylopectin), owing to its specificity for alveolar macrophages. It has been shown that this is a promising strategy for the treatment of pulmonary tuberculosis [123]. New inhalable antitubercular drug delivery systems using rifampicin or isoniazid, among others, based on the use of liposomes, micro-, and nanoparticles offer a promising

treatment for tuberculosis, with important advantages, such as fewer drug doses or low dosing frequency. However, some problems such as deposition of the inhaled formulation in the lungs need to be solved [180].

The incorporation of rifampicin in DL-PLG microspheres [158] affords sustained release over 7 hours, the concentration in murine and human macrophages increasing and toxicity decreasing. Owing to their small size, it is possible to accomplish pulmonary administration with aerosols [159, 160], improving efficacy with lower sistemic toxicity in a tuberculosis animal model.

5.2. Brucellosis

Brucellosis is an infection that usually affects livestock but is also able to infect humans through contact with infected animals. Because of its intracellular location, long treatments with several antibiotics are required. Relapses are frecuent owing to the low efficacy of many drugs and the lack of patient compliance [61].

Recently, rifampicin-loaded mannosylated dendrimers have demonstrated specific pH-dependent delivery of this antibiotic to rat alveolar macrophages [81].

Gentamicin, encapsulated in different types of liposomes [54], has been evaluated against murine monocytes infected with *B. abortus*. All such liposomes reduced the number of bacteria, the most effective being SPLVs (stable plurilamellar vesicles). Microspheres of PLA/PLGA containing gentamicin [30, 61] show good activity against *S. aureus*, without cytotoxicity. Particles of suitable size can be obtained affording controlled release at the site of infection, enabling their use in intracellular infections such as brucellosis. PLGA nanoparticles containing gentamicin are accumulated *in vitro* in murine monocyte cell lines, and also show selective distribution in the liver and spleen of mice, with potential applications in the treatment of brucellosis [155, 156].

5.3. Salmonellosis.

Bacteria of the genus *Salmonella* are facultative intracellular parasites that cause salmonellosis and typhoid fever. Antibiotics effective against this type of

bacteria have limitations owing to problems of formulation, low penetration, or the appearance of side effects; these can be solved by using carrier systems.

Mice infected with *S. dubliniensis* have been treated with gentamicin encapsulated in liposomes and in free form, survival increasing with the encapsulated antibiotic. The number of bacteria decreases in the spleen and organs usually invaded in this type of infection, such as mesenteric lymph nodes and Peyer plaques [^{11}6].

Liposomal ciprofloxacin, administered intravenously and intraperitoneally to mice infected with intracellular *S. typhimurium*, has increased residence time in plasma and the concentration of drug in the liver, spleen, lungs and kidneys is also increased, while when administered intratracheally its pulmonary retention is increased. Compared with free ciprofloxacin, it prolongs survival and reduces the number of bacteria in the liver and spleen [120].

In vitro studies carried out in mouse macrophages infected with *S. typhimurium* (residing in the phagosome) and recombinant *S. typhimurium* (residing in the cytosol) have reported increased cellular penetration and biological activity of gentamicin formulated in liposomes composed of DOPE–N-succinyl-DOPE-PEG, especially against pathogens residing in the cytosol, since the presence of anionic lipids such as DOPE (dioleylphosphatidylethanolamine) destabilises the structure of the liposome at the endosomal pH, favouring its fusion with the endosome membrane and releasing the drug to the cytosol [25]. The distribution of the antibiotic incorporated in these carriers is also altered in mice, the concentration in the kidneys decreasing and that in the liver and spleen increasing, leading to a reduction in the numbers of bacteria in these organs [52].

Similar results have been obtained using [^{3}H]ampicillin-loaded polyisohexylcyanoacrylate nanoparticles, which permit greater cellular penetration and antibacterial activity in murine macrophages infected with *S.typhimurium* [157].

5.4. Lysteriosis

Lysteria monocytogenes is a facultative intracellular parasite able to cause meningitis and septicaemia. The encapsulation of ampicillin in liposomes decreases the survival of *L. monocytogenes* in mouse peritoneal macrophages to different extents, depending on the composition of the liposomes [127].

Gentamicin formulated in liposomes containing DOPE and sensitive to pH has been reported to increase the concentration of drug in mouse macrophages infected with *L. monocytogenes*, increasing its bactericidal activity. This formulation is more effective against *L. monocytogenes* than against other bacteria owing to its location in the cytosol [25].

5.5. Fungal infections

Fungal infections, especially candidiasis and aspergillosis, are one of the main causes of morbidity and mortality in immunocompromised individuals and frequently appear as hospital-acquired infections. Some fungi are facultative parasites. There are many drugs that can be used to combat such infections, but the intracellular location of the pathogens means that high doses are required to reach therapeutic levels inside the cell, leading to toxic effects [42, 137].

Polyene antibiotics are the most widely used owing to their broad spectrum of antifungal activity. Amphotericin B is the drug of choice against systemic infections (candidiasis, aspergillosis or histoplasmosis) and pulmonary aspergillosis. The drug is accumulated in phagocytic cells, increasing the antifungal activity of macrophages and PMNs [181]. Its main limitation is its dose-dependent toxicity, especially in the kidneys [137, 182, 183]. Different drug delivery systems have been designed for amphotericin B, mainly based on the use of liposomes, lipid complexes and colloidal dispersions that are active against a broad range of fungi, and allow the administration of higher doses, reducing the potential nephrotoxicity of amphotericin B [184, 185].

Owing to its liposolubility, amphotericin B is usually encapsulated in liposomes. The administration of amphotericin B and liposomal amphotericin B for the treatment of systemic candidiasis in mice affords similar results as regards survival, but the liposomal form allows the administration of higher doses, with better results [130, 131, 183]. The presence of high concentrations of the drug in the liver, spleen and lung [131] is an advantage, since these are the organs most frequently affected by disseminated infections. Similar results have been reported for experimental cryptococcosis [132] and histoplasmosis [133].

The incorporation into a carrier of macrophage activators together with the antibiotic may improve the results. The use of amphotericin B in tuftsin-liposomes, as compared with liposomal amphotericin B, prolongs the survival of mice infected with *A. fumigatus* and fully erradicates the infection [42, 129]. Similar results have been achieved with amphotericin B incorporated in tuftsin-bearing liposomes, which showed better anticryptococcal activity in a murine model [134].

Currently, there are several lipid formulations of amphotericin B available commercially. The therapeutic index of Ambisome (SUVs), Amphocil or Amphotec (a colloidal dispersion) and Abelcet (a lipid complex) is increased with respect to the conventional formulation of amphotericin B (Fungizone) [161, 186-188]. The differences in their structure and composition mean that their distribution in the organism will be different. Owing to their relatively large molecular size, Amphocil and Abelcet are rapidly taken up by the organs of the RES, especially the liver and lung, while Ambisome attains high plasma levels over long periods of time. Another commercial formulation of amphotericin B is Fungisome, made of liposomes of lecithin and cholesterol. Clinical trials have demonstrated that in renal transplantation patients Fungisome does not produce any toxicity and is effective in fungal infections resistant to fluconazole and conventional amphotericin B [189].

The intracellular activity of Ambisome in rat peritoneal macrophages infected with *C. albicans* is lower than that of Fungizone owing to the slow release of the drug inside cells [190]. However, in different animal models the greater efficacy and

safety of Ambisome as compared with free amphotericin B and other treatments are widely documented, as is its capacity to reach tissues such as the brain, lungs and kidneys [191].

Comparative studies of the three lipid formulations show that they are more efficacious than Fungizone and that they allow the use of higher doses, without toxicity. In a model of systemic cryptococcosis, Ambisome and Amphocil showed similar efficacy, higher than that of Abelcet, in increasing survival and decreasing the fungal load in brain, kidney and lung. Ambisome was more effective in the liver and Amphocil in spleen against infection due to *C. albicans* in the central nervous system of rabbits. Ambisome and Fungizone had similar efficacies, higher than that of Amphocil and Abelcet [143, 192].

The encapsulation of complexes of amphotericin B and cyclodextrins in liposomes obtained *in situ* from a preliposomal mixture affords greater stability in plasma and its therapeutic efficiency is greater than that of free amphotericin B or traditional liposomes in animals with aspergillosis and cryptococcosis [135].

The use of pegylated liposomes decreases the binding of plasma proteins to the liposome surface and hence phagocytosis, the time of residence in blood increasing and the concentration in the organs of the RES decreasing. This favours accumulation in other organs. Nevertheless, cellular penetration decreases, and they are therefore less effective against intracellular *C. albicans* in murine peritoneal macrophages [136].

The inclusion of specific ligands at the liposome surface increases cellular penetration. Ligands such as PAM (p-aminophenyl-mannopyranoside) direct the drug better to the liver and spleen, while OPM (O-palmitoyl mannan) does so to the lung, such that both would be useful in pulmonary mycoses. Additionally, PAM and OPM have been reported to achieve elevated levels of drug over long periods of time in albino rats [137].

Amphotericin B encapsulated in nanospheres of poly(ε-caprolactone) covered with poloxamer 188 and in mixed micelles of poloxamer 188 has been shown to increase concentrations in liver and spleen, with less renal toxicity than Fungizone in rats [161]. Their activity against intracellular *C. albicans* -both *in vitro* and *in vivo* in neutropenic mice- is less than that of amphotericin B, but they are less toxic, meaning that higher doses can be administered [162]. The complexing of amphotericin B with the polysaccharide arabinogalactan decreases its toxicity. Survival is similar to that obtained with Ambisome in mice with candidiasis and cryptococcosis, with a greater reduction in the number of CFUs in kidney and brain at elevated doses [167]. Lipid nanospheres based on a lipid emulsion of amphotericin B show good antifungal efficiency in a rat model of candidiasis [163, 164].

The activity of nystatin is similar to that of amphotericin B, although some strains are resistant to this and susceptible to nystatin. The use of nystatin is limited owing to its toxicity and problems of administration through the parenteral route [183]. The formulation of nystatin in MLVs increases its activity against fungal infections *in vitro*, its toxicity to human erythrocytes decreasing [139, 141, 142]. In animal models of candidiasis, it has been described to increase the maximum doses tolerated, prolonging survival [140], and to remove the pathogen form the liver, kidneys, spleen, lungs and brains, and it is less toxic than amphotericin B [143]. Against disseminated aspergillosis in neutropenic mice, it has been shown to prolong survival, reducing the fungal load in liver and kidney [144]. Against pulmonary aspergillosis in neutropenic rabbits, it prolongs survival and reduces tissue damage, although to a lesser extent that amphotericin B, with tolerable signs of renal toxicity [143]. In a murine model, pH-sensitive liposomes of nystatin showed better activity than neutral liposomes against *Cryptococcus neoformans* infections [145]. A commercial form is available –Nyotran- that allows i.v. administration and maintains the therapeutic activity of nystatin [146, 183]. Similar

results, with an increase in anti-cryptoccocal activity, have been obtained using negatively charged liposomes containing chloroquine [147].

5.6. Leishmaniasis

This disease is endemic to many countries and causes many deaths if not treated appropriately. Currently, the number of infected patients has increased owing to co-infection with HIV. It is caused by protozoans of the genus *Leishmania*, and is transmitted through vector insects. *Leishmania* are obligate intracellular parasites that cause infection only in the phagolysosome of host macrophages. There are four clinical syndromes: visceral, cutaneous, mucocutaneous and diffuse cutaneous leishmaniasis [42, 77, 166].

There are many drugs available with anti-*Leishmania* activity, but their use is limited owing to their toxicity or the appearance of resistance. The drugs of choice are pentavalent antimonials such as sodium stibogluconate, or meglumine antimoniate, but these are toxic for the liver and skin. It is also possible to use amphotericin B and pentamidines, but these are toxic to the heart and liver. Other drugs with anti-*Leishmania* effects are also available, such as aphidicolin, atovaquone, primaquine, plant substances such as amarogentin [151] and andrographolide [28], and cytotoxic drugs, such as doxorubicin and methotrexate [154].

Owing to the location of this parasite the use of carrier systems is very helpful, as is the inclusion of specific ligands on the surface of the cell, such as sugars [28, 150] or peptides such as tuftsin [42, 53, 128] or fMLP [152], which are also macrophage activators. The use of immunoliposomes, which at their surface include antibodies against specific antigens of the parasite, increases selectivity for infected cells [154]. Additionally, immunoliposomes favour the access of drugs to organs such as the bone marrow, the gastrointestinal tract, and the skin, which harbour the microorganisms mainly responsible for relapses, especially in immunodepressed patients [42, 138]. The activity of sodium stibogluconate

encapsulated in nyosomes [193] or in tuftsin-liposomes [42, 53] is increased both *in vitro* and *in vivo* in hamsters, better results being obtained with lower doses.

Amphotericin B has also been tested in liposomal form against *Leishmania*. A comparative study of the four existing formulations of amphotericin B demonstrated better effectiveness of all of them in comparison with sodium stibogluconate in mice with visceral leishmaniasis, the dose tolerated increasing in the three lipid forms against cutaneous leishmaniasis. Ambisome and Amphocil administered intravenously reduce the size of the lesion, while Fungizone and Abelcet are inactive. Administered subcutaneously, Ambisone is ineffective [194, 195]. In comparison with traditional amphotericin B and meglumine antimoniate, Ambisome shows greater effectiveness in the elimination of *L. infantum* in mouse liver, spleen and lungs. It also allows the use of higher doses, achieving a greater and more prolonged effect [196]. Ambisome, Amphocil, Abelcet and nyosomal amphotericin B provided good results against visceral leishmaniasis in mice in parasite clearance from the liver, spleen and bone marrow, except Abelcet, which was inactive in bone marrow [197]. In mice with acute infections, sodium stibogluconate in nyosomes is more effective at eliminating parasites from the liver, spleen and bone marrow than Ambisome, Amphocil and Abelcet, providing active levels of the drug in tissues for long periods of time [148]. A good *in vitro* uptake of pentavalent antimony by mouse peritoneal macrophages infected with *Leishmania chagasi* was obtained using negatively charged phosphatidylserine liposomes [149].

Different clinical trials carried out with liposomal amphotericin B and amphotericin B-lipid complex have demonstrated good efficacy in immunocompetent patients with visceral leishmaniasis [198-202].

Amphotericin B in tuftsin-liposomes improves efficacy in comparison with the free form and with liposomes without the peptide both *in vitro* and *in vivo* in animal models, achieving a greater reduction in parasite burden in the spleen [53, 128]. Different liposomal formulations of amphotericin B have been shown to have greater

efficacy than that of the free form and greater accumulation in liver and spleen in clinical and experimental studies [138, 203].

Liposomal pentamidine isethionate increases efficacy in the elimination of parasites in the hamster spleen. The inclusion of sugars (mannose) at the surface of the liposomes increases efficacy, without the appearance of toxic effects in the liver [150].

Amarogentin encapsulated in liposomes and nyosomes improves the elimination of parasites from the spleen in hamsters [151]. The efficiency of andrographolide in liposomes and mannose-liposomes is improved and the drug shows less toxicity to the hamster liver and kidneys than in free form [28]. Doxorubicin-bearing immunoliposomes increase the activity of liposomes and free doxorubicin in macrophages infected by *L. donovani*, with no toxicity. Studies carried out in mice have confirmed better efficiency, spleen infections being fully eliminated. Liposomes and immunoliposomes do not have cardio- or hepatotoxicity [154]. fMLP-liposomes direct primaquine to macrophages *in vitro.* In hamsters, they show higher efficacy in removing spleen parasites, with no toxicity [152].

The bioavailability of aphidicolin formulated as a nanosuspension is improved when administered orally, its *in vitro* activity against intracellular *L. donovani* increasing in murine macrophages [166]. *In vitro* Amphotericin B conjugated with arabinogalactan is effective against *L. major* and *L. infantum*. Administered intravenously to mice infected with *L. major*, it is more effective than Fungizone in delaying the appearance of lesions. Subcutaneous administration permits higher doses to be given [75]. The penetration of NPC1161 (8-aminoquinolone) bound to HPMA (hydroxypropylmethacrylamide) containing N-acetylmannosamine increases penetration in mouse peritoneal macrophages, with no signs of toxicity. Its *in vivo* efficacy is similar to that of the free drug [77].

5.7. HIV Infections.

The monocyte-macrophage system is the first to be infected by HIV. It supports the intracellular replication of the virus by acting as a reservoir, which favours the dissemination of the infection and protects the virus against antiretroviral treatment [87, 204].

There is a broad range of drugs available that are active against HIV, but they have insufficient macrophage penetration capacities and low bioavailability, giving rise to the appearance of resistance and toxic effects due to accumulation in other organs [57]. The use of carrier systems allows doses to be lowered. The ability of these vehicles to convey drugs into cells is increased because macrophages infected by HIV show greater phagocytic activity than those that are healthy [165, 205, 206].

To be active, nucleoside-analogue reverse transcriptase inhibitors must be phosphorylated to 5'- triphosphate by cell kinases. Administration of the phosphorylated form is not possible because of the low cell membrane permeability to nucleotides and its rapid hydrolysis in biological fluids [153]. Their encapsulation in carrier systems may overcome this obstacle.

The encapsulation of Stavudine (d4T) in different kind of liposomes, improves the penetration into human macrophages in all cases [27]. The phosphorylated form of dideoxycytidine (ddCTP) in liposomes reduces infection in the spleen and bone marrow of infected mice, the signs of the disease decreasing, with no modifications in blood parameters [153]. The incorporation of molecules such as sCD4 and CD4-IgG at the surface of liposomes increases selectivity towards infected macrophages [207].

Zalcitabine and saquinavir formulated in nanoparticles achieve efficacious concentrations *in vitro* in infected human macrophages. However, when encapsulated zalcitabine shows no improvement over the free drug, which is able to accumulate in macrophages. In nanoparticles, the antiviral activity of saquinavir is

improved in both acute and chronic infection in human macrophages [55]. The administration to rats, both orally and intravenously, of azidothymidine (AZT) formulated in nanoparticles increases the bioavailability of the drug and increases its concentration in blood, in the brain, and in organs of the RES [56, 165]. Covering the particles with polysorbate 80 alters the distribution of the drug, concentrations falling in the liver and increasing in the blood and in the brain. This is advantageous, since the brain is one of the viral targets of most difficult access [56].

The AZT-tuftsin complex is able to inhibit viral replication in T cells, and it increases antigen presentation and the release of IL-1 in mouse peritoneal macrophages, such that it could be of use for transporting the drug to macrophages [208].

The active form of dideoxycytidine (ddCTP) encapsulated in erythrocytes inhibits HIV replication in infected human and cat macrophages and protects uninfected cells [172, 173]. It decreases the degree of splenomegaly, lymphadenopathy and hypergammaglobulinaemia in mice and cats [172, 173] without altering blood parameters. Sometimes, prodrugs that will give rise to the drug upon degradation by erythrocyte enzymes are encapsulated. This is the case of the AZTp2AZT dinucleotide encapsulated in erythrocytes, which affords controlled release of AZT over long periods of time [174].

Glutathione (GSH) is an antioxidant agent able to inhibit viral replication. Alternate treatment with fludarabine and AZT produces better results when GSH-erythrocytes are administered together with the AZT [209]. Infected mice treated with AZT+ddI+GSH-erythrocytes show a greater reduction in proviral DNA in bone marrow and brain than with ddI +GSH [204, 210].

A common observation in patients with AIDS is the appearance of opportunistic infections due to viruses, such as herpes simplex (HSV). Drugs such as adefovir (PMEA) or acyclovir (ACV) inhibit the proliferation of both HIV and HSV. The encapsulation of PMEA in erythrocytes increases the inhibition of HIV and HSV

in human monocytes/macrophages, without toxicity [169]. Encapsulation of heterodinucleotide prodrugs such as bis-PMEA or ACVpPMPA provides better protection against HIV and HSV in human macrophages and for longer periods of time [170, 171].

Lamivudine-loaded mannosylated poly(propyleneimine) dendrimers have significantly increased cellular uptake by macrophage cell lines *in vitro* in comparison with the free drug [80].

Acetylated low-density lipoproteins (LDL) have been used as vehicles for shuttling AZT to phagocytic cells. Owing to the hydrophilic nature of AZT, a prodrug -5'-O-13-oxymyristate-AZT was used. Uptake is much greater in both mouse macrophages and human monocytes [87]. Flucytosine (FLT) and azidothymidine (AZT) bound to LDLs inhibit viral replication in human macrophages, but not in lymphocytes, since these do not have scavenger receptors [168].

6. Concluding remarks.

In the past decade many advances have been made in the field of delivery systems containing antibiotics against different intracellular infections. Of special importance is the progress made in the field of liposomes, whose efficacy has been demonstrated in the treatment of intracellular infections in studies carried out with cell lines, animal infection models and, in particular, in different clinical trials. This efficacy has been demonstrated in infections with mycobacteria, leishmaniasis, and -especially- in fungal infections. Other delivery systems, such as conjugated nanoparticles or lipid systems have also been studied, with the finding of increases in intracellular uptake and antibacterial action but with as yet limited clinical evidence. Special mention should be made of biological carrier systems such as cell ghosts, which -owing to their high biocompatibility and their specific delivery to phagocytic cells- seem to be promising delivery systems for antibiotics, antifungals and antiretroviral agents. Nevertheless, the possible clinical implications of antibiotic

delivery systems active against intracellular infections remain little explored in pathologies such as brucellosis, salmonellosis, lysteriosis and AIDS.

Acknowledgements.

This work was supported by the Spanish National I+D+i Plan (Projects: PI060152 and SAF2001-0740).

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CAPÍTULO 3

Recent advances in delivery systems for anti-HIV1 therapy

Journal of Drug Targeting (2007) 15, 21-36

Recent advances in delivery systems for anti-HIV1 therapy

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Abstract

In the last years, different non-biological and biological carrier systems have been developed for anti-HIV1 therapy. Liposomes are excellent potential anti-HIV1 carriers that have been tested with drugs, antisense oligonucleotides, ribozymes and therapeutic genes. Nanoparticles and low-density lipoproteins (LDLs) are cell-specific transporters of drugs against macrophage-specific infections such as HIV1. Through a process of protein transduction, cell-permeable peptides of natural origin or designed artificially allow the delivery of drugs and genetic material inside the cell. Erythrocyte ghosts and bacterial ghosts are a promising delivery system for therapeutic peptides and HIV vaccines. Of interest are the advances made in the field of HIV gene therapy by the use of autologous haematopoietic stem cells and viral vectors for HIV vaccines. Although important milestones have been reached in the development of carrier systems for the treatment of HIV, especially in the field of gene therapy, further clinical trials are required so that the efficiency and safety of these new systems can be guaranteed in HIV patients.

Keywords: HIV1 therapy, carrier systems, drug delivery, gene therapy

Introduction

In recent years, the struggle against HIV/AIDS has enhanced the development of different therapeutic strategies, involving two approaches: research and development of new anti-HIV drugs and gene therapy through the use of DNA vaccines. Anti-HIV drugs, often known as antiretroviral drugs, are the most frequent alternative to establish control over the reproduction of the virus and to guarantee slow progression of the disease. Antiretroviral drugs are classified in four main groups: nonnucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs) and fusion inhibitors. These drugs are usually used in combined therapy, such as in the case of highly active antiretroviral therapy (HAART). HAART therapies still have important limitations such as the high cost of the treatments, the adverse effects of some antiretroviral drugs, drug resistance, drug interactions, non-compliance problems, etc. (Kalkut 2005).

Gene therapy has been proposed as an alternative for human immunodeficiency disease (HIV1) (Luque et al. 2005; Dropulic and June 2006; Von Laer et al. 2006). Gene therapy for HIV1 infection is based on two main strategies. The first uses lethal genes to kill the infected cells before the virus can produce infective particles, while the second strategy involves protecting the cell from the virus (Luque et al. 2005).

RNA-based strategies for anti-HIV therapy based in the use of antisense oligonucleotides, small interfering RNA, RNA decoys and ribozymes are currently in progress, including combinatorial gene therapy strategies (Akkina et al. 2003; Nielsen et al. 2005; Ramezani et al. 2006).

Antisense oligonucleotides are short sequences of nucleic acids complementary to a given messenger nucleic acid (sense sequence). Antisense oligonucleotide therapy permits the inhibition of gene expression in HIV infection. The problems associated with this type of therapy essentially derive from the short halflife of antisense oligonucleotides and the difficulty involved in accessing the

inside of the cell. Many modifications have been made for oligonucleotide which can significantly improve the half-life, stability and cellular uptake. Toxic effects such as mild thrombocytopenia and hyperglycemia, complement activation and coagulation cascades and hypotension are all associated with the use of antisense therapy (Zelphati et al. 1994; Putnam 1996; Jason et al. 2004; Phillips 2005).

Small interfering RNA or silencing RNA (siRNA) are a class of 20-25 nucleotide-long RNA molecules that interferes with the expression of a specific gene. siRNA may protect stem cells from HIV infection (Lamothe and Joshi 2000; Akkina et al. 2003; Song et al. 2003). RNA decoys inhibits the capacity of HIV1 Tat protein for the transcription of HIV1 genome (Bohjanen et al. 1997). Ribozymes are RNA molecules with catalytic activity. Combinatorial use of rybozymes in cultured cells have recently demonstrated the supression of HIV1 replication (Ikeda et al. 2006).

HIV/AIDS vaccines are an interesting alternative under investigation since currently no vaccine has been approved for routine clinical use. Basically, there are two types of HIV vaccines: the preventive type, whose main objective is to prevent HIV infection and therapeutic vaccines, which aim to improve the immune system in HIV-positive patients. Outstanding in this field are the canarypox vaccines or the DNA vaccines, among others (Dorrell 2005; Lambert 2005; Tubiana et al. 2005).

Drugs with potent anti-HIV1 activity *in vitro* are ineffective or have a limited activity *in vivo* due to limited uptake in target cells. Different biological and non-biological delivery systems have been developed in order to solve this problem. In recent years, non-biological carrier systems, such as liposomes, nanoparticles, LDL or peptides and biological carriers, such as viral vectors, erythrocyte ghosts, stem cells or bacterial ghosts that permit specific *in vivo* intracellular delivery of anti-HIV1 drugs, have been developed with a view to improving their cellular uptake and enhancing their activity. Delivery systems allow the therapeutic agent to be vectors to organs of the reticuloendothelial system (RES), where the virus often resides and multiplies. In this way, it is possible to reduce the doses administered, improving

efficiency and decreasing toxicity. The ability of such carriers to vehicle drugs into cells is increased by the fact that HIV-infected macrophages show greater phagocytic behaviour than those that are not infected (Löbenberg et al. 1998). Delivery systems can also be used in the field of gene therapy, especially with antisense oligonucleotides (Figure 1) and with anti-HIV vaccines (Cockrel and Kafri 2003).

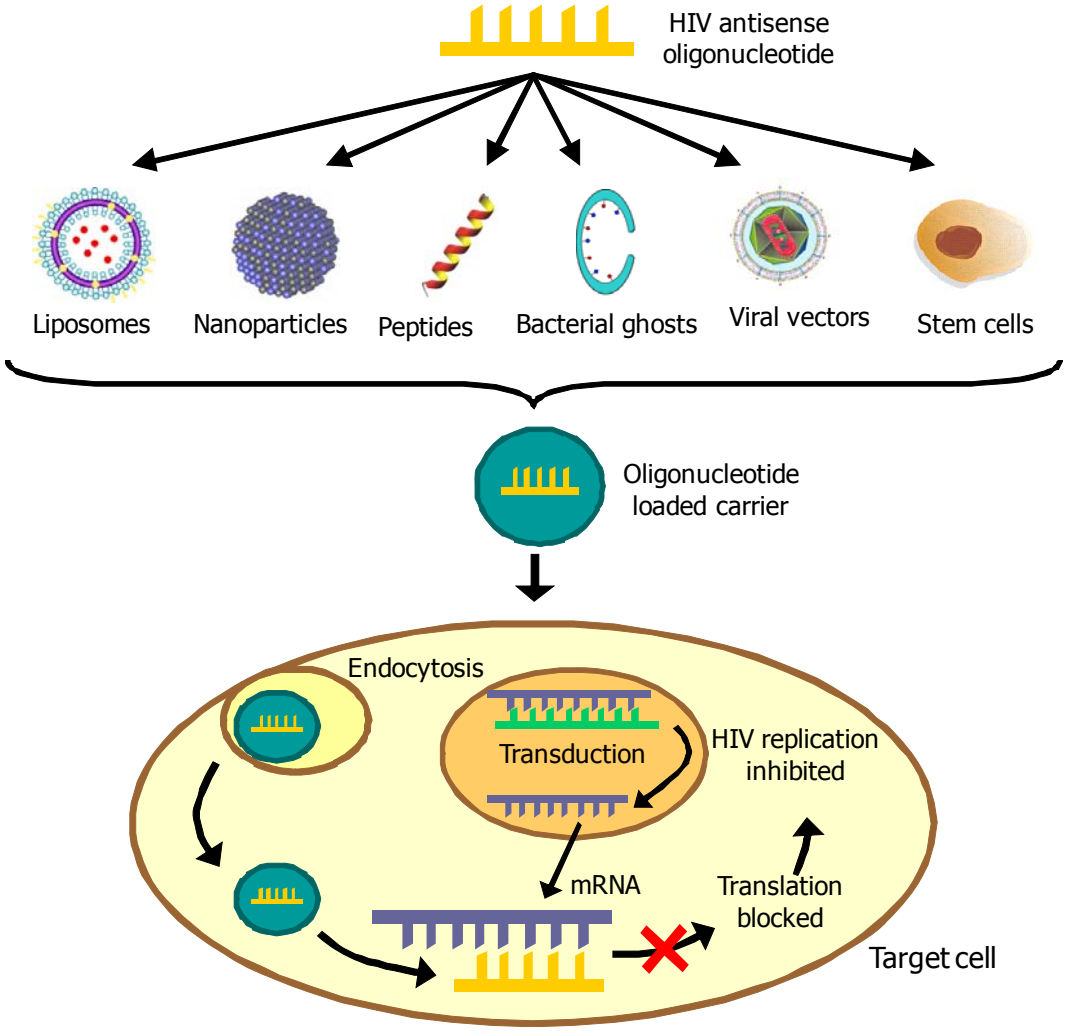

Figure 1. Carrier systems for antisense oligonucleotide therapy.

Table 1 shows the non-biological and biological delivery systems for drugs and genetic material developed for anti-HIV1/AIDS therapy. The aim of this review is to discuss recent advances in non-biological and biological delivery systems used with drugs and genetic material in anti-HIV therapy, their advantages, limitations and potential clinical applications.

Non biological carrier systems		
Carrier	***Encapsulated drug***	***Reference***
Liposomes		
Anionic Liposomes	Stavudine (d4T)	Katragadda et al. 2000
	ddCTP	Oussoren et al. 1999
	Zidovudine	Jin et al. 2005
Cationic Liposomes	Anti-HIV Rev-binding aptamer, ribozymes	Konopka et al. 1998a, Konopka et al. 1998b
	Ribozymes	Kitijima et al. 1997
Immunoliposomes	Rev antisense phosphorothioate oligonucleotide	Selvam et al. 1996
	Rev antisense phosphodiester or phosphorothioate oligonucleotide	Zelphati et al. 1993, Zelphati et al. 1994
	Env region antisense RNA	Renneisen et al. 1990
	Indinavir	Gagne et al. 2002, Desormeaux et al. 2005
Sterically Stabilized Liposomes	Indinavir	Gagne et al. 2002, Desormeaux et al. 2005
Nanoparticles	Zalcitabine, Saquinavir	Bender et al. 1996
	Zidovudine	Löbenberg et al. 1997, Löbenberg et al. 1998
	DNA vaccines	Cui and Mumper. 2003
Low Density Lipoproteins (LDL)	5'-O-13-oxamyristate-AZT	Hu et al. 2000
	Flucytosine, AZT	Mankertz et al. 1996
Peptides	Oligonucleotides	Lochmann et al. 2004
	AZT	Fridkin et al. 2005, Giammona et al. 1998, Giammona et al. 1999
	Ganciclovir, acyclovir	Cavarallo et al. 2004

Table 1. Classification of carrier systems used for anti-HIV1 therapy.

Biological carrier systems		
Carrier	***Encapsulated drug***	***Reference***
Erythrocytes	ddCTP	Magnani et al. 1992, Magnani et al. 1995
	AZTp2AZT	Benatti et al. 1996
	GSH	Fraternale et al. 2001, Fraternale et al. 2002, Fraternale et al. 2003
	PMEA	Perno et al. 1997
	Bis-PMEA	Rossi et al. 2001
	ACVpPMPA	Franchetti et al. 2000
Viral Vectors		
Poxviruses	Recombinant HIV1 antigens vaccines	Slyker et al. 2005
	Canarypox ALVAC-HIV vaccines	Goepfert et al. 2005
	Recombinant fowlpox virus vaccines	Coupar et al. 2005
Adenoviruses	Plasmid DNA vaccines	Xin et al. 2005, Casimiro et al. 2003
	rAAV/SIV vaccines	Johnson et al. 2005
Retroviruses	gag-pol, env genes	Marusich et al. 2005
Stem Cells	Anti-HIV ribozyme	Amado et al. 1999
	Transdominant mutant Rev protein	Mautino et al. 2001, Su et al. 1997
	RRE-anti-HIV1 gene	Kohn et al. 1999
	REVNL3-huM10, REVNL3-Fx genes	Podsakoff et al. 2005
Bacterial Ghosts	DNA vaccines	Ebensen et al. 2004, Walcher et al. 2004, Szostak et al. 1993

Table 1 (cont.). Classification of carrier systems used for anti-HIV1 therapy.

Cellular targets for anti-HIV1 therapy

CD4+ cells are the primary targets for HIV infection, but HIV infects and replicates in many other cell types in the body, including other T cells or macrophages. Other cells, like dendritic cells, CD8+ or B lymphocytes and neutrophils have been found to bind and transfer infectious virus to these target cells (Levy 2002).

HIV infection starts with adsorption of a HIV virion to the cell membrane, through specific interaction with the cell surface receptor CD4 that is expressed mainly in T cells and monocyte-macrophages. It constitutes an interesting cellular target and several CD4 mimic molecules as well as small molecular weight drug named BMS-378806 have been developed to inhibit infection of the cell (Reeves and Piefer 2005).

However, HIV is also able to infect CD4- cells. The mechanism is not yet known, although there have been different hypothesis. Some authors have proposed that the presence of a membrane protein, claudin-7 on the target cells increase viral susceptibility and may have an important role in the infection of this type of cells (Zheng et al. 2005).

The chemokine receptors CCR5 or CXCR4 are the major coreceptors for HIV1 entry. CCR5 is an attractive target since it was described that human subject carrying a 32 base pair deletion in CCR5 are resistant to HIV infection (Huang et al. 1996; Liu et al. 1996; Samson et al. 1996). Some molecules that inhibit these co-receptors are being developed, like the current leading CCR5 antagonists vicriviroc, maravirok and aplavirok (Dorr et al. 2005; Reeves and Piefer 2005; Strizki et al. 2005). Also, several peptides and small molecular compounds that are inhibitors of the CXCR4 co-receptor have been assayed (Altmeyer 2004).

Among several other cellular proteins, cyclin-dependent kinases (cdks) are required for replication of HIV1. Pharmacological cdk inhibitors have proven to have potent *in vitro* activity against HIV1, like flavopiridol and roscovitine. However, other cellular proteins that are known to be required for viral replication may be good targets for antiviral drugs (Gibbs and Sorensen 2000; Zala et al. 2000; Tamet al. 2001), like cyclophilin (CYPA) an isomerase that is specifically incorporated into newly formed HIV1 virions (Sokolskaja and Luban 2006). Those cellular proteins showing upregulation of expression during viral infection should be analysed as potential targets for antiviral drugs (Fruh et al. 2001).

The host proteome offers also numerous potential therapeutic targets. To identify cellular proteins that may play a role in infection, the effect on the trans-activating transcriptional activator (Tat) on cellular gene expression was analysed and a list of gene products that may serve as potential therapeutic targets for the inhibition of viral replication was compiled, including PouAFI (OBF-1), complement factor H related 3, CD4 receptor, ICAM-1.NA and Cyclin AI (Liang et al. 2005; Shang 2002).

Also some intrinsic antiretroviral factors that restrict HIV infections have been identified as the TRIM 5α in mouse cells and Ref1 antiretroviral resistance factor in humans. It remains to be determined if variants of this proteins may have therapeutic utility for HIV infection (Hatziioannou et al. 2004; Keckesova et al. 2004; Yap et al. 2004).

Non-biological delivery systems

Conventional drug delivery systems such as liposomes and nanoparticles, among others, play an important role in drug therapy and especially in the field of cancer therapy (Ranade and Hollinger 2004). Among other advantages, this type of system allows the efficiency of the active ingredient to be increased; it reduces toxicity and modifies the pharmacokinetic behaviour of the drug and in particular, it facilitates selective access of the drug to its site of action, which is sometimes intracellular.

Non-biological carrier systems such as liposomes, nanoparticles, LDL and different types of peptides have been assayed as potential carriers of drugs and genetic material for anti-HIV therapy.

Liposomes

Liposomes are a classic non-biological delivery system used with different drugs and have many therapeutic applications. Liposomes are defined as microscopic vesicles formed by one or several phospholipid bilayers surrounding an

aqueous compartment, with a particle size between 25 nm and several microns, such that they are able to encapsulate hydrophilic drugs inside the aqueous phase, or hydrophobic drugs bound to or incorporated in the lipid bilayer. They are usually made up of phospholipids, natural or synthetic and cholesterol and they may incorporate other lipids and derivatives and also proteins. Among the advantages of liposomes is the fact that they are biodegradable, sparingly toxic and immunogenic and they show a high drug/vehicle ratio (Agrawal and Gupta 2000; Kozubek et al. 2000). However, they also have some disadvantages, such as their poor stability, both in the bloodstream, due to the presence of serum lipoproteins, and in storage; there is also the problem of the low encapsulation efficiency of the methods used to elaborate them, the presence of solvent residues in the final preparation, which is unacceptable owing to their possible toxicity and the high costs of industrial production (Prior et al. 2002).

When liposomes are administered *in vivo*, they are rapidly eliminated from the circulation by monocytes and macrophages and are accumulated in cells of the RES, especially the liver and spleen. This characteristic of liposomes is an advantage in anti-HIV therapy because macrophages are an important reservoir of HIV. The existence of negative charges favours the binding of serum proteins to the lipid surface, in turn favouring phagocytosis and increasing the resistance to serum HDL and hence providing stability in the blood stream. Also, cationic liposomes show greater monocyte activation. The electrostatic attraction between the positive charges of the liposome and the negative charges of the cellular surface may also favour certain liposome internalization mechanisms, such as fusion or endocytosis (Vitas et al. 1996).

Cholesterol or sphingomyelin are often included in the composition of liposomes in order to increase the rigidity of the lipid bilayer, which hinders interactions with the cell. Accordingly, the presence of increasing amounts of cholesterol will decrease the cellular penetration of the vesicles (Katragadda et al. 2000; Ahsan et al. 2002). Cholesterol also favours liposome stability against serum

lipoproteins and the increase in the time of circulation in the blood and it decreases the percentage of encapsulation of the drug because it modifies the thickness of the membrane (Karlowsky and Zhanel 1992; Vitas et al. 1996).

There are essentially four types of liposomes: anionic liposomes, cationic liposomes, immunoliposomes and sterically stabilised liposomes. All these types are candidates for use as carriers of drugs and genetic material in anti-HIV therapy.

To be pharmacologically active, antiretroviral drugs that are inhibitors of transcriptase must be phosphorylated to 5'-triphosphate by cellular kinases. Owing to the poor ability of some cells to phosphorylate these compounds, the alternative would be the administration of the phosphorylated form directly. However, this is not possible because of the low permeability of cellular membranes to nucleotides and their rapid hydrolysis in biological fluids (Oussoren et al. 1999). The inclusion of these drugs in liposomes has proved to be a good alternative in the treatment of AIDS. Stavudine (d4T) is a transcriptase inhibitor. Its incorporation in liposomes with different compositions in all cases elicited an increase in cellular penetration in human macrophages (Katragadda et al. 2000). In infected mice, the triphosphorylated form of dideoxycytidine (ddCTP) incorporated in liposomes reduces the content of proviral DNA in spleen and bone marrow. Signs of the disease, such as hypergammaglobulinaemia, lymphadenopathy and spleenomegaly decreases, with no modification to any blood parameters (Oussoren et al. 1999). The incorporation onto the surface of liposomes of molecules such as soluble CD4 and CD4-IgG, which inhibit HIV infectivity, decreases the selectivity to infected macrophages (Raulin 2002).

Zidovudine (AZT) is a very useful drug in antiretroviral therapy but it has several secondary effects. AZT shows dose-dependent toxic effects, especially in bone marrow. At high concentrations, it may also cause anaemia and leucopenia. Another problem with AZT is its short half-life, which means that administrations must be frequent in order to maintain its plasma levels within the therapeutic range.

The use of AZT drug delivery systems allows sustained plasma levels to be maintained.

Recent pharmacokinetic studies carried out in the rat with zidovudine myristate-loaded liposomes administered intravenously have shown a sustained release effect characterised by an increase in drug plasma levels and in the area under the curve of AZT when administered in liposomes in comparison with administration in aqueous solution. At the same time, the administration of AZT incorporated into liposomes allowed a better accumulation of the drug in the RES (Jin et al. 2005).

Cationic liposomes as carriers are safe and nonimmunogenic for *in vivo* gene delivery. Cationic liposomes are characterised by the fact that the cationic components of the outside interact with the negatively charged molecules of DNA, producing a system able to carry genetic material. In this type of liposome, the positive charge of the lipid allows interaction with the cell surface, permitting the selective release of genetic material to the target cell, reaching the nucleus and allowing expression of the therapeutic gene.

Cationic liposomes containing antisense oligonucleotides and ribozymes facilitate cytoplasmic delivery to target cells for the inhibition of gene expression and protect anti-HIV1 drugs from nuclease digestion. The cellular uptake of ribozymes complexed with cationic liposomes was higher than naked ribozymes and ribozymes complexed with anionic liposomes, although this type of carrier of genetic material has some problems *in vitro*, since the antiviral effect of the liposome-complexed ribozyme was not sequence-specific, limiting its potential applications *in vivo* (Kitajima et al. 1997; Konopka et al. 1998a,b; Duzgunes et al. 2001). Recent studies *in vitro* using a T cell line PBMC's infected with HIV1 and *in vivo* using mouses carried out with cationic liposomes containing phosphorothioate oligonucleotides demonstrate a enhanced cellular uptake both *in vitro* and *in vivo* expressed as the inhibition of the production of p24 antigen (Miyano-Kurosaki et al. 2004).

Immunoliposomes are characterised by having on their surface specific antibodies or fragments of antibodies and in this way they promote a specific action. Many studies *in vitro* and *in vivo* in experimental animals have demonstrated the inhibition of the expression of HIV1 using antibody-targeted liposomes containing antisense oligonucleotides or anti-HIV1 drugs (Zelphati et al. 1993; Selvam et al. 1996; Bestman-Smith et al. 2000; Gagne et al. 2002; Desormeaux and Bergeron 2005). Preliminary *in vitro* studies using antisense RNA encapsulated in protein A-bearing liposomes inhibited HIV expression. Protein A-liposomes were directed to target cells using anti-CD3 monoclonal antibodies. This study demonstrated that a dose-dependent inhibition of HIV1 replication in anti-CD3- treated cells could be achieved using a env-coding RNA in the antisense orientation while the same RNA sequence in the sense orientation was not effective (Renneisen et al. 1990). Later studies carried out *in vitro* with liposomes containing antisense nucleotides revealed that this type of liposome may inhibit viral replication in acutely and chronically HIV1-infected cells in tissue culture. In acute infection, the increase in the anti-HIV effect is related to the delivery of the antisense oligonucleotide carried in liposomes in the cell cytoplasm and in chronic infection the increase in the effectiveness of the therapeutic compound is related to its delivery to the nucleus (Zelphati et al. 1994).

Another type of liposome, known as sustained circulation liposome, is characterised by a significant increase in the half-life of the liposome. Within this group of liposomes, the so-called "sterically stabilised liposomes" are important. These incorporate polyethyleneglycol (PEG) at the surface of the liposome, which provides them protection against phagocytosis by mononuclear cells, prolonging the time of circulation of the drug delivery. Combined use of sterically stabilised liposomes and immunoliposomes elicits an important increase in the selectivity of anti-HIV drugs for lymphoid tissues. Studies with indanavir incorporated into sterically stabilised immunoliposomes have shown that these liposomes are very specific *in vitro* and studies performed in mice have demonstrated a greater

accumulation of the drug in lymphoid tissues over at least 15 days after injection of the liposomes (Gagne et al. 2002; Desormeaux and Bergeron 2005).

Nanoparticles

Nanoparticles are stable, solid, polymeric particles with a size range between 10 and 1000 nm that are able to incoporate drug in their interior or bound to the polymeric matrix (Bender et al. 1996; Löbenberg et al. 1998; Von Briesen et al. 2000). They are formed of biodegradable polymers of natural or synthetic origin which are degraded *in vivo* either by an enzymatic route or a non-enzymatic action or a combination of both, giving rise to non-toxic products that are readily removed from the organism through the usual metabolic pathways. The natural polymers used are bovine serum albumin, human serum albumin, collagen, gelatine and haemoglobin, although their use is limited owing to their high cost and doubtful purity. Accordingly, it currently is more common to see the use of synthetic polymers, owing to their elevated biodegradability and biocompatibility, such as polyamides, polyamino acids, polyalkyl-acyanoacrylates, polyesters, polyorthoesters or polyurethanes (Jain 2000).

The nanoparticles of polymeric systems have been tested to improve the efficiency or antiretroviral drugs. Zalcitabine and saquinavir were formulated in nanoparticles and tested *in vitro* in HIV-infected human macrophages. In both cases, effective drug concentrations were achieved inside the cells. However, zalcitabine incorporated into nanoparticles did not show any improvement with respect to the free drug since this latter does penetrate macrophages. In contrast, saquinavir in nanoparticles elicited important improvements in antiviral activity in both chronic and acute infection (Bender et al. 1996). The distribution throughout the organism of AZT formulated in nanoparticles was studied after oral and i.v. administration to rats (Löbenberg et al. 1997, 1998). In both cases, encapsulation in nanoparticles implied an increase in bioavailability and also in the concentration of the drug in blood, brain and organs of the RES rich in macrophages, such as the liver, lungs, spleen and bone marrow in comparison with the drug in solution.

Coating the particles with polysorbate 80 modified the nanoparticles distribution, the drug levels decreasing in liver and increasing in blood and in the brain. This is an advantage because the brain is one of the targets of the virus that is very hard to access with currently available antiretrovirals (Löbenberg et al. 1998). Nanoparticles constituted by complex of antisense oligonucleotides and their phosphorotioate analogues with protamine enhanced the cellular uptake of oligonucleotides and the inhibitory effect on HIV1 transactivation in primary human macrophages and Jurkat cells (Dinauer et al. 2004). Microparticles and nanoparticles have also been proposed as future delivery systems for DNA vaccines (Cui and Mumper 2003).

Low density lipoproteins (LDL)

Plasma LDLs are quasispherical endogenous nanoparticles with a diameter of approximately 22 nm, formed by a lipid nucleus surrounded by a phospholipid monolayer containing cholesterol and apoprotein B-100 (apo B) (Kader et al. 1998). Their metabolism involves internalisation by means of LDL receptors present on almost all cells of the organism, through the lipoprotein Apo B present in the LDL and in cells belonging to the monocytes-macrophage system by means of scavenger receptors (Mankertz et al. 1997; Hu et al. 2000; Von Briesen et al. 2000). Chemical modifications typical of the metabolism of Apo B, glycosylation, acetylation, derivatisation to aldehyde and oxidation, alter the affinity of LDLs for scavenger receptors of phagocytic cells. Owing to their lipid nature, they are useful for the transport of lipophilic drugs. Accordingly, the binding of anti-HIV drugs to modified LDLs is a strategy for transporting these substances to the inside of phagocytic cells (Hu et al. 2000).

Acetylated LDLs have been used as carriers for AZT to phagocytic cells. Owing to the hydrophilic nature of AZT, a prodrug- 5'-O-13-oxamyristate-AZT- was used; this is more lipophilic and more effective than AZT. Cellular uptake was much greater than with the free drug both in mouse J774 macrophages and in human U937 monocytes (Hu et al. 2000). Flucytosine (FLT) and AZT bound to LDLs

inhibited the replication of the virus in human macrophages but not in lymphocytes since the latter do not have scavenger receptors (Mankertz et al. 1996).

Peptides

Peptides have been proposed as carriers of drugs and genetic material because they are able to penetrate the lipid barrier of cell membranes and release their load of therapeutic agents intracellularly in the cytoplasm or nucleus. These peptides, known as protein transduction domains (PTD) or cell-permeable peptides (CPP) may be of natural or synthetic origin. Peptides for cell transduction usually contain lysine or arginine as the Tat from HIV1. The passage of peptides across the cell membranes is accomplished through a process of protein transduction, delivering the therapeutic agents inside the cell. The mechanism of protein transduction inside the cell is an electrostatic interaction with the plasma membrane, penetration by macropinocytosis and release to the cytoplasm and nuclei (Hyndman et al. 2004; Futaki 2005; Gupta et al. 2005; Noguchi and Matsumoto 2006).

The use of protein transduction for anti-HIV therapy involves the transduction of a protein which has been engineered with a target site for HIV protease. Therefore, this protein will only become activated in cells containing HIV protease but will remain inactive in uninfected cells (Vocero-Akbani et al. 1999). Antisense oligonucleotide or interfering RNAs (siRNAs) are also potential candidates to be used in combination with PTD to increase stability, enhance cellular uptake and improve anti-HIV therapy (Noguchi and Matsumoto 2006).

Fusogenic peptides have provided an improvement in gene transfection efficiency (Vaysse et al. 2000). Antisense oligonucleotides such as oligodeoxynucleotides and oligophosphorothioates coupled to an influenza-derived fusogenic peptide increase cellular delivery and antiviral activity *in vitro* using CEM-SS lymphocytes infected by HIV1. At the same time, the covalent attachment of the fusogenic peptide to the oligonucleotide increased its resistance to enzymatic degradation (Lochman et al. 2004). This fusogenic peptide was derived from the

influenza virus haemagglutinin envelop glycoprotein (Remeta et al. 2002). Recently, it has been suggested that fusion peptides inserted into T cells membrane may inhibit antigen-specific T cell proliferation and cytokine secretion *in vitro* (Gerber et al. 2004; Quintana et al. 2005).

Tuftsin (L-threonyl-L-lysyl-L-prolyl-L-arginine) is a tetrapeptide located in the Fd fragment of the gamma-globulin molecule, which is recognized by specific receptors of phagocytic cells, mainly macrophages. As well as showing specific affinity for macrophages, the peptide tuftsin also has activator properties typical of phagocytic cells. Tuftsin may be used as carrier of anti-HIV1 drugs in AIDS therapy with potential clinical applications (Fridkin et al. 2005). The synthesis of conjugates of Tuftsin with anti-HIV1 drugs uses solution methodology (Fridkin et al. 2005). Conjugates of Tuftsin with 3'-azido-3'-deoxythymidine (AZT) inhibits reverse transcriptase activity and HIV1 antigen expression and increases the processing and presentation of antigens and the release of IL-1 in mouse peritoneal macrophages *in vitro* (Fridkin et al. 2005).

Studies addressing the cellular uptake of antisense oligonucleotides conjugated with cell-penetrating model peptides have proposed that the enhanced biological activity of antisense oligonucleotides after derivatization with membrane-permeable peptides may be related to an increased affinity for target structures such as nucleic acids or proteins as well as to an improved membrane translocation (Oehlke et al. 2002).

Another alternative is the use of macromolecular prodrugs, which are made by covalent linking of inert macromolecules with a drug, obtaining a molecular prodrug with a change in the pharmacokinetic properties of the conjugate with respect to the free drug. An example is the use of glycosylated macromolecular conjugates of antiviral drugs with a polyaspartamide (Giammona et al. 1998, 1999; Cavallaro et al. 2004). Macromolecular prodrugs of AZT with α,β-poly(N-2-hydroxyethyl)-DL-aspartamide (PHEA) allow a sustained action effect of the antiretroviral drug to be achieved (Giammona et al. 1999).

Biological delivery systems

Biological carrier systems are an alternative to the more classical non-biological carrier systems, such as liposomes or nanoparticles, which are increasingly used. Within the different biological carrier systems, of great interest are cells and cell ghosts, which are compatible systems from the biological point of view and which are able to provide the sustained release and specific delivery of drugs and genetic material to tissues, organs and cells. Biological carrier systems such as, erythrocyte ghosts, bacterial ghosts, viral vectors and more recently, genetically engineered stem cells can be suitably manipulated and loaded with drugs or genetic material, permitting specific drug delivery *in vivo* with therapeutic application in anti-HIV therapy (Lanao and Sayalero 2006).

Erythrocyte ghosts

Erythrocytes account for the greater part of cells in the blood and are the main transporters of oxygen to cells and tissues in the organism. When erythrocytes age, they undergo a series of catabolic changes that lead to a loss of cell flexibility. These changes favour cellular lysis in the circulation or phagocytosis by the RES, especially by the spleen, where the erythrocytes are attacked by lysosomal enzymes that lead to breakage of the cell membrane and the degradation of haemoglobin.

Erythrocytes are potential biocompatible vectors for different bioactive substances, including drugs and proteins. Erythrocyte ghosts can be prepared in different ways, usually by hypotonic dialysis (Millán et al. 2004). They find application as drug reservoirs that permit a sustained release of the drug to the organism or for selectively carrying the drug to the RES, especially the liver, spleen and bone marrow. This latter application is of special importance in anti-HIV drugs because they are able to improve the penetration of such drugs into phagocytic cells to a considerable extent.

To increase the phagocytosis of carrier erythrocytes a frequent strategy is to modify membrane proteins, as in the case of the transmembrane band 3 protein,

with substances such a zinc, the peptide melitin, the dye acridine orange or the oxidising agents phenylhydrazine and diamine, followed by stabilisation with an agent that fosters cross linking such as bis(sulfosuccinimidyl) suberate (BS3). This favours opsonisation by IgG and C3b, increasing phagocytosis by macrophages (Magnani et al. 1992; Perno et al. 1997; Fraternale et al. 2003).

Erythrocytes display a series of advantages that make them suitable as carrier systems for shuttling drugs into phagocytic cells of the RES. Important aspects are that they are easily handled, they have high biological compatibility and stability and they provide the possibility of achieving a sustained action effect of the encapsulated drug. Erythrocytes also allow the encapsulation of peptides with biotechnological applications (Grimaldi et al. 1997; Bax et al. 1999; Magnani et al. 2002; Hamidi and Tajerzadeh 2003; Millán et al. 2004; Rossi et al. 2005).

The use of erythrocytes as carrier systems does however have some drawbacks: the rapid elimination of erythrocytes by the RES, which may lead to toxicological problems; the rapid leakage of certain substances from the erythrocyte, which hinders their access to the RES; difficulties in preparation and storage since they are biological carriers (Álvarez et al. 1995; Jain and Jain 1997; Moss et al. 2000; Sugai et al. 2001; Valbonesi et al. 2001; Millán et al. 2004; Rossi et al. 2005). One of the main therapeutic applications of carrier erythrocytes is in the field of anti-HIV therapy. Anti-HIV peptides such as nucleoside analogues successfully inhibit the replication of immunodeficiency viruses. Considering the importance of the monocyte-macrophage system in infection by HIV1, the specific delivery of these therapeutic peptides into macrophages, which act as an important reservoir for the virus, is of huge therapeutic interest.

The encapsulation of antiretroviral drugs in erythrocytes has been studied in some depth. The active form of ddCTP encapsulated in erythrocytes inhibits HIV replication in infected macrophages and protects uninfected cells from infection (Magnani et al. 1992, 1995). In *in vivo* studies, it decreased spleenomegaly, lymphadenopathies and hypergammaglobulinaemia, with no alterations in

haematological parameters in mice and in cats (Magnani et al. 1992, 1995). Sometimes the encapsulation of prodrugs is used; this leads to the release of the drug since they are degraded by erythrocyte enzymes. This is the case of the dinucleotide AZTp2AZT encapsulated in erythrocytes, which is able to provide a controlled release of AZT over long periods of time (Benatti et al. 1996).

Glutathione is an antioxidant agent able to inhibit viral replication. Its encapsulation in erythrocytes could be very useful for the protection of macrophages challenged by HIV infection in combination with other antiretroviral drugs. The use of AZT as a protector of uninfected macrophages and lymphocytes, combined with fludarabine to eliminate infected lymphocytes, used alternatively, has afforded promising results in infected mice. However, the administration of GSH in erythrocytes together with AZT improved the efficiency of the treatment (Fraternale et al. 2001). Infected mice were treated with AZT + DDI and AZT + DDI + GSH-erythrocytes. The reduction in the content of proviral DNA in bone marrow and brain decreased significantly in mice treated with AZT + DDI + GSH-erythrocytes (Fraternale et al. 2002, 2003).

In patients with AIDS, it is common to find opportunistic viral infections, such as herpes simplex (HSV), which also invades macrophages and increase HIV replication. There are antiviral drugs such as adefovir (PMEA) or acyclovir (ACV) that inhibits the proliferation of both HIV and HSV. PMEA encapsulation in erythrocytes achieved a better inhibition of HIV and HSV in human monocytes/macrophages than free PMEA, with no signs of cytotoxicity (Perno et al. 1997). Prodrugs are also encapsulated, as dinucleotides, and these are converted into the active form of the drug by endogenous enzymes. The encapsulation of bis-PMEA (Rossi et al. 2001) or ACVpPMPA (Franchetti et al. 2000) exerted a greater degree of protection against HIV and HSV and for longer periods of time than did the free drug.

Biological carrier systems in HIV gene therapy

Gene therapy for the treatment of AIDS is an interesting but as yet underdeveloped therapy that aims to replace CD4+ and other immune cells by cells

genetically engineered to resist virus replication (Amado et al. 1999; Fanning et al. 2003). The success of gene therapy against HIV1 depends on good functioning of the different components of the immune system, such as the thymus or bone marrow and the use of an appropriate gene able to render the cell resistant to infection by HIV1. Antisense oligonucleotides and ribozymes can be targeted to specific sites within the HIV genome (Poeschla et al. 1996). The use of small RNA molecules that produce gene inactivation is a frequently used tool in gene therapy (Nielsen et al. 2005). In this field, antisense RNAs and ribozymes are designed to inhibit cellular or HIV RNA function (Lamothe and Joshi et al. 2000).

New gene therapy strategies based in combinatorial therapies for the treatment of HIV using series of RNA-based inhibitors have demonstrated to be effective in reducing viral loads. Recent papers demonstrate that a combined gene therapy strategy using antisense RNA and ribozymes using viral vectors to target different sites within the HIV1 RNA in a CD4+ T lymphoid cell line or CD34+ -derived monocytes provides enhanced inhibition of HIV1 infection. (Li et al. 2005; Ramezani et al. 2006).

A promising gene for HIV gene therapy is RevM10. Rev protein is a virally encoded sequence-specific RNA-binding protein (Pollard and Malim 1998). The binding of Rev to the RER and the transport of RER containing the RNA to the cytoplasm is essential for viral replication. RevM10 is a dominant negative form of the Rev trans-activator protein that efficiently inhibits human immunodeficiency virus (HIV) (Gottfredsson and Bohjanen 1997; Veres et al. 1998; Hamm et al. 1999). Studies carried out in cultured T cell lines and primary T cells have shown that relatively high steady-state levels of RevM10 protein are required to achieve the inhibition of HIV replication (Plavec et al. 1997).

The use of nucleic acid vaccines offers a promising technique for the development of prophylactic or therapeutic vaccines based on the use of DNA plasmids to induce immune responses by direct administration of DNA-encoding

antigenic proteins to animals and this is also suitable for the induction of cytotoxic T cells (Donnelly et al. 1997, Felnerova et al. 2004; Lanao and Sayalero 2006).

Currently in the field of gene therapy for the prevention and treatment of HIV/AIDS gene carriers are being developed that encapsulate and protect the nucleic acid and selectively release the vector/nucleic acid complex to the target tissue so that the genetic material will later be released at cellular level. In practice, there are three ways to achieve this aim. The first is through the use of modified viruses containing the genetic material of interest. The second alternative is to use living cells modified genetically, such as stem cells, to deliver transgenic material into the body. The third alternative is the use of bacterial ghosts that encapsulate the genetic material and allow its specific delivery to phagocytic cells (Lanao and Sayalero 2006).

Viral vectors as gene delivery systems. Attenuated or modified versions of retroviruses, adenoviruses, lentiviruses, Sendai virus, herpes virus, adeno-associated viruses and poxviruses can all be used as carriers, with different therapeutic applications, although their use has been questioned (El-Aneed 2004; Tomanin and Scarpa 2004; Klink et al. 2004). The main advantage of the use of viruses as gene delivery vehicles is their high transduction efficiency *in vivo*. Retroviral transduction efficiency has been enhanced using ultrasonic standing-wave fields to facilitate the retroviral transduction rate (Lee and Peng 2005). However, gene delivery systems viruses have some drawbacks since they only deliver genes to cells during mitosis and they are sensitive to lysis by complement when they are administered to patients. Furthermore, viruses may mutate; they are limited in the size of gene they can carry and they may give rise to immune complications (Falkner and Holzer 2004; Klink et al. 2004).

Delivery systems based on the use of viruses as carriers have recently found application in the fields of HIV vaccines (Humeau et al. 2004; Excler 2005; Duerr et al. 2006). Poxviruses are a heterogenous group of DNA viruses that are potentially safe because they are unable to replicate in humans. Poxviruses have shown their

ability to induce mucosal immune responses against foreign expressed antigens, which is important for the development of vaccines against mucosal pathogens, as happens with HIV (Gherardi and Esteban 2005; Moroziewicz and Kaufman 2005). Recent clinical trials in HIV1-exposed infants using recombinant modified Ankara virus vaccinia (MVA) as a vector to deliver recombinant HIV type 1 (HIV1) antigens (MVAHIVA) using HIV1-specific CD8+ cell responses suggest that stimulation with the MVAHIVA may be useful in the evaluation of vaccine receivers (Slyker et al. 2005). Recent clinical trials in healthy uninfected adults using canarypox ALVACHIV vaccines have revealed high reactogenicity associated with an increased dose of the vaccine (Goepfert et al. 2005). Clinical trials and experiments carried out in mice and macaques using recombinant fowl pox virus vaccines directed against HIV1 subtype B, against HIV1 subtype AE, or SHIV have shown that the relevant proteins are expressed (Coupar et al. 2006).

Adenovirus type-5 (Ad5)-based vaccines produce an adequate T cell immune response in baboons and monkeys, although the hepatocellular tropism of this viral vector limits their safety (Casimiro et al. 2003; Xin et al. 2005). Adequate immune responses were recently obtained in macaques using an adeno-associated virus vector vaccine (Johnson et al. 2005).

Other viruses such as alpha-viruses, flaviruses, rhabdoviruses, myxovirus, paramoxyviruses or picorna virus may be used as potential vaccine vectors for anti-HIV therapy (Figure 2) (Excler 2005).

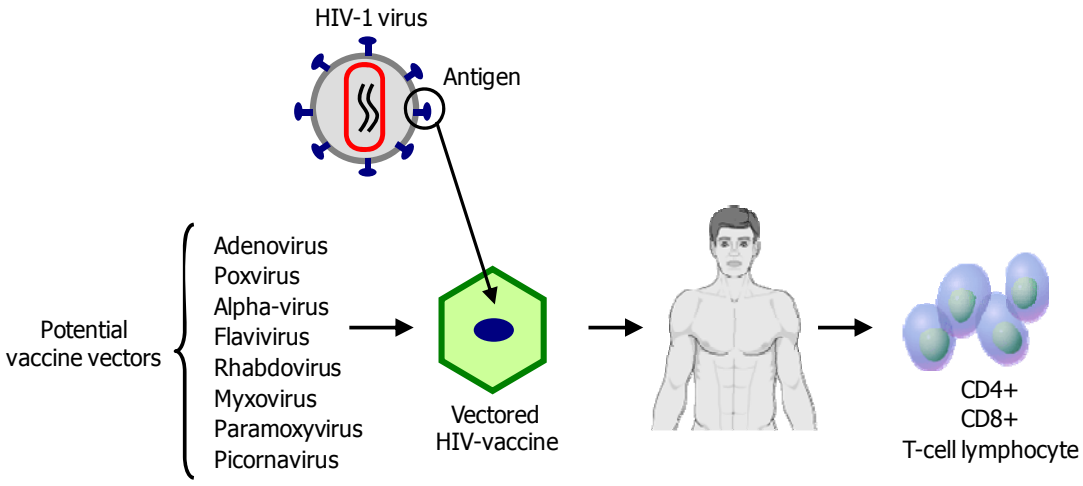

Figure 2. Viral vectors for HIV-1 vaccines.

Recently, the "intracellular immunization" strategy has been tested. This is based on gene transfer using a spleen necrosis virus (SNV) as a retroviral vector with potential applications in future human gene therapy applied to anti-HIV1 treatment. *In vitro* experiments using human haematopoietic cells demonstrated the reduction of HIV1 replication by the SNV retroviral-vector carrying therapeutic transgenes in a range of 4-10-fold in T-lymphocytes, primary human PBMCs and macrophages (Marusich et al. 2005).

Stem cells. Another alternative is to use living cells modified genetically, such as stem cells, to deliver transgenic material into the body. The use of stem cells as delivery systems is a novel and attractive technique in the field of gene therapy in which the cells of the patients themselves are genetically engineered in order to introduce a therapeutic transgene and then used to deliver the genetic material (Becker 2005; Lanao and Sayalero 2006). Haematopoietic stem cells may be used as a drug delivery system of antiviral genes with a view to reducing HIV1 replication.

Considering the affinity of primitive CD34+ haematopoietic stem cells for HIV host cells, stem cells can be collected and transduced *in vitro* by spinoculation

(O'Doherty et al. 2000) with a retroviral or a lentiviral vector (Bai et al. 2003; Brenner and Malech 2003) expressing the trans-dominant mutant rev protein RevM10, which inhibits HIV1 replication. Autologous engineered haematopoietic stem cells administered *in vivo* allow the delivery of genetic material to target cells such as monocytes or macrophages, suppressing HIV1 replication (Amado et al. 1999; Mautino et al. 2001) (Figure 3).

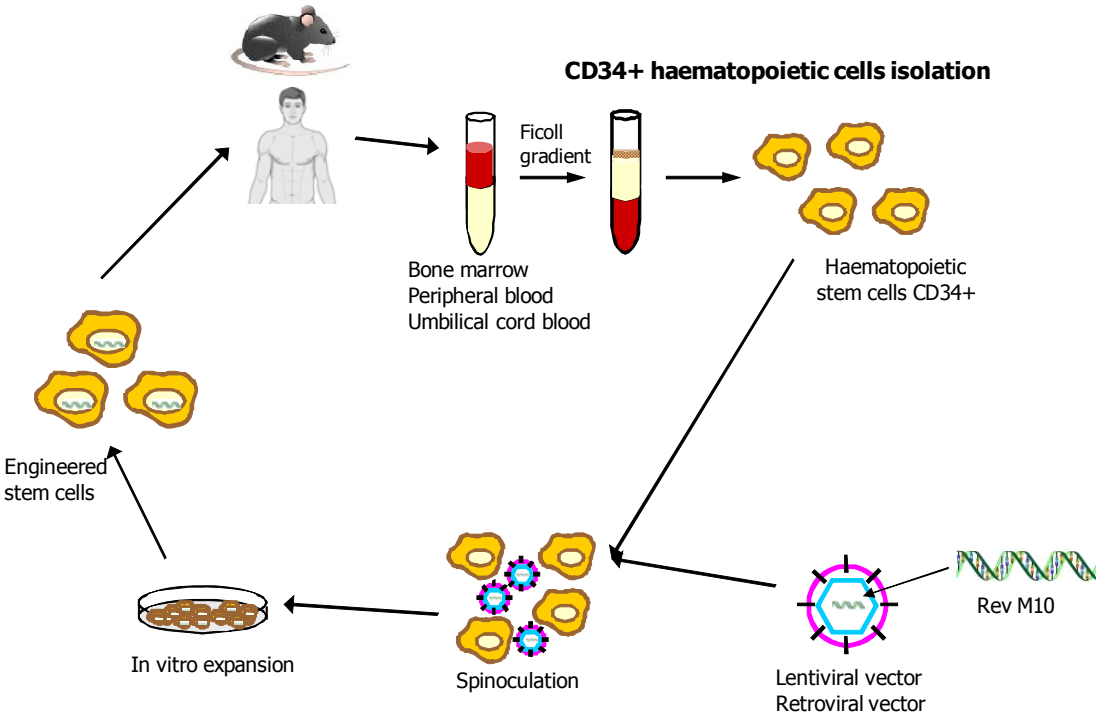

Figure 3. Use of engineered haematopoietical stem cells for anti-HIV therapy.

The use of haematopoietic stem cells has allowed antiviral genes to be introduced into both T cells and macrophages for the treatment of AIDS (Bonyhadi et al. 1997; Morel et al. 1999; Chan et al. 2005; Van Griensven et al. 2005). *In vitro* and *in vivo* studies have shown the potential usefulness of haematopoietic stem cells for anti-HIV1 therapy (Siapati et al. 2005). Preliminary experiments *in vivo* after reconstitution of human bone marrow implanted in mice with transduced

haematopoietic stem cells with RevM10 have revealed an increase in the myeloid progenies expressing RevM10 (Su et al. 1997).

Clinical studies performed in paediatric AIDS patients using bone marrow haematopoietic CD34+ stem cells isolated and transduced by a retroviral vector as carriers of an RRE anti-HIV1 gene and reinfused in the patients showed that gene containing leukocytes in the peripheral blood were seen only at a low level and only at the start of treatment (Kohn et al. 1999; Bauer et al. 2000). More recent clinical trials carried out by the same group in 2 paediatric HIV1 patients using two retroviral vectors, one encoding a dominant-negative REV protein (REVNL3-huM10) and another encoding a non translated marker gene (REVNL3-FX), showed that following cell transduction and reinfusion of the CD34+ bone marrow stem cells, gene-marked cells were present at low levels and persisted for only a few months. In one of the patients, the reappearance of peripheral blood mononuclear cells containing the huM10 gene suggested selective survival for the peripheral blood cells containing this gene (Podsakoff et al. 2005).

Although the use of stem cells to inhibit HIV is a promising technique in the field of gene therapy, there are some drawbacks that remain to be solved. Examples are altered haematopoiesis and thymopoiesis during HIV1 infection, or the need to increase the amount of transduced haematopoietic stem cells, among others (Van Griensven et al. 2005).

Bacterial ghosts. Bacterial ghosts are intact, non-living, non-denatured bacterial cell envelopes devoid of cytoplasmic contents that are created by lysis of bacteria but that maintain their cellular morphology and native surface antigenic structures (Lanao and Sayalero 2006). Bacterial ghosts are produced by protein E-mediated lysis of Gram-negative bacteria (Marchart et al. 2003). Bacterial ghosts allow the encapsulation of drugs, proteins and therapeutic DNA or RNA, allowing specific delivery to different tissues and cell types and they have a good capacity for being captured by phagocytic cells, and antigen-presenting cells such as dendritic cells. The main disadvantages of this type of delivery system are the possibility that they

might revert to being virulent, the possibility of lateral gene transfer, the stability of the recombinant phenotype and pre-existing immunity against the vector used (Tabrizi et al. 2004).

Bacterial ghosts are a promising delivery system for DNA vaccines (Ebensen et al. 2004; Tabrizi et al. 2004; Walcher et al. 2004; Mayr et al. 2005). Although there is little experience in the use of bacterial ghosts in HIV1 therapy, in experiments carried out using animal models with piglets, mice and rabbits using bacterial ghosts of *K. pneumoniae*, *S. typhymurium* and *E. coli* as carriers of HIV1-RT and HIV1 gp41 as antigen targets it was observed that these ghosts induced humoral and cellular immune responses (Szostak et al. 1993, 1996; Walcher et al. 2004; Mayr et al. 2005).

Concluding remarks

In the past ten years, many advances have been made in the development of both non-biological and biological carrier systems in anti-HIV therapy. In the field of non-biological carrier systems, liposomes have been developed to carry drugs such as stavudine, dideoxycytidine or AZT that allow the specific delivery of these drugs to infected macrophages. Assays have been made in immunoliposomes containing antisense RNA. Advances have also been made in the field of nanoparticles, LDL and peptides containing anti-HIV drugs and genetic material destined for gene therapy. However, despite such progress most studies carried out have been limited to the *in vitro* situation or in experimental animals and there is very little clinical information about this type of carrier in humans. On the other hand, important advances have been made in the use of biological carrier systems for the treatment of HIV using erythrocyte ghosts, bacterial ghosts, viral vectors and engineered stem cells.

Of special interest is the progress made in the field of HIV gene therapy through use of autologous stem cells and viral vectors. These have led to important

milestones in the development of HIV vaccines in clinical assays carried out all over the world. In the near future, it would be desirable to see a boost in clinical trials with new carrier systems in HIV patients.

Acknowledgements

This paper was supported by the National Research and Development Plan. Ministry of Science and Technology (Project SAF 2001-0740).

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CAPÍTULO 4

Increasing the selectivity of amikacin in rat peritoneal macrophages using carrier erythrocytes

Enviado para su publicación a *European Journal of Pharmaceutical Sciences*

Increasing the selectivity of amikacin in rat peritoneal macrophages using carrier erythrocytes.

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Abstract

The selectivity of amikacin in macrophages *in vitro* and its biodistribution in peritoneal macrophages and other tissues were studied in rats using carrier erythrocytes. Amikacin-loaded erythrocytes were prepared using a hypotonic dialysis method. The *in vitro* uptake of amikacin by peritoneal macrophages was studied using cell monolayers. The *in vivo* uptake by macrophages and the tissue distribution of amikacin were studied in two groups of rats that received either amikacin in saline solution, or amikacin-loaded erythrocytes. Pharmacokinetic analyses were performed using model-independent methods. The administration of the antibiotic using carrier erythrocytes elicited a higher accumulation in macrophages, both *in vitro* and *in vivo*. The tissue pharmacokinetics of amikacin *in vivo* using carrier erythrocytes revealed an accumulation of the antibiotic in specific tissues such as the liver and spleen. Minor changes in the pharmacokinetics were observed in organs and tissues such as renal cortex and medulla. According to the partition coefficients obtained, the relative uptake of amikacin when carrier erythrocytes were used was: spleen > peritoneal macrophages > liver > lung > renal cortex > renal medulla. Loaded erythrocytes can be seen to be potentially useful for the delivery of aminoglycoside antibiotics in macrophages.

Key words: Amikacin, carrier erythrocytes, pharmacokinetics, macrophages, biodistribution.

1. INTRODUCTION

Phagocytic cells are the first line of defence of the host against infections owing to their capacity to uptake, kill, and remove the pathogen responsible for the infection (Ernst, Stendahl, 2006). Nevertheless, some infectious diseases are caused by facultative and obligate parasites that are able to survive and multiply once inside the phagocytic cells. Different mechanisms are used by microorganisms to avoid the bactericidal action of phagocytes, and through them they are able to reside in different subcellular compartments (Reinoso et al, 1995; Ahsan et al., 2002).

The intracellular location of microorganisms protects them against host defences and against the action of antibiotics that are unable to penetrate into cells, making these infections difficult to treat (Holmes et al., 1966; Carryn et al., 2003). In addition, phagocytic cells can act as reservoirs for facultative parasites, often leading to relapses and chronic disease (Carryn et al., 2003; Klempner, 1984). Monocytes and macrophages are the most frequent reservoirs owing to their higher phagocytic capacity, less potent microbiocidal properties and longer half-lives (Maurin, Raoult, 2001).

Among the microorganisms that cause intracellular infections there are bacteria such as *Mycobacterium tuberculosis, M. Leprae, Brucella, Listeria, Legionella pneumophila, Salmonella, Chlamydia psittaci,* viruses such as *HIV, influenza virus*, *Herpes simplex*, fungi such as *Candida albicans* or *Aspergillus fumigatus*, and parasites such as *Leishmania*.

The penetration of antibiotics into cells is not sufficient for them to be effective against intracellular pathogens. The antibiotic must also reach the subcellular compartment containing the microorganisms and must remain active in the intracellular environment (Carryn et al., 2003; Barcia-Macay et al. 2006). Often, high antibiotic doses are necessary to achieve effective concentrations in cells, which may result in toxic effects that make their use unsuitable (Lutwyche et al., 1998; Schiffelers et al., 2001).

In order to solve these problems, a common strategy is the encapsulation of antibiotics in carrier systems that will be taken up by phagocytic cells, delivering the antibiotic directly to the target cell (Lanao et al., 2007; Briones et al., 2008). Among these systems, colloidal carriers such as liposomes (Ahsan et al., 2002; Schiffelers et al., 2001; Karlowsky, Zhanel, 1992) and micro/nanoparticles (Ahsan et al. 2002; Bender et al., 1996) are those most frequently used. However, biological carriers such as erythrocytes have advantages that make them more suitable for such purposes. Thus, they show higher biocompatibility and stability than other carrier systems, and they allow controlled release of the encapsulated drug and hence a higher accumulation in the organs of the reticuloendothelial system (RES). Moreover, phagocytosis can be improved be means of different modifications to the erythrocyte membrane (Gutiérrez et al., 2004a; Magnani et al., 2002; Eichler et al., 1986).

Carrier erythrocytes have been used previously as carrier systems for different anti-infective agents such as ciprofloxacin (Mishra et al., 1996), gentamicin (Eichler et al., 1986), tetracycline (DeLoach, Wagner, 1984), and the anti-*Leishmania* agents homidium bromide (DeLoach, 1985) and formycin A (Berman, Gallalee, 1985), showing a higher selectivity of drug distribution, especially in the organs of the RES.

Amikacin is an aminoglycoside antibiotic that is effective against infections due to Gram(-) bacteria, but owing to its high solubility in water it penetrates poorly into cells, and its clinical use has been limited because of its toxic effects: mainly nephro- and ototoxicity (Maurin, Raoult, 2001).

The aim of the present work was to study amikacin uptake by rat peritoneal macrophages *in vitro* and *in vivo*, and the changes that occur in its plasma pharmacokinetics and tissue distribution when it is administered encapsulated in carrier erythrocytes through the intraperitoneal route.

2. MATERIAL AND METHODS

2.1. Amikacin encapsulation in rat erythrocytes

The housing and experimental treatment of the animals were in accordance with current Spanish and European Union legislation and complied with the "Principles of Laboratory Animal Care".

The encapsulation of Amikacin in rat erythrocytes was accomplished using a hypotonic dialysis method (Eichler et al., 1986; Gutiérrez et al., 2004b; Gutiérrez et al., 2005). Briefly, fresh blood was obtained from male Wistar rats by retro-orbital puncture and centrifuged to remove plasma. Packed erythrocytes were washed twice with Hanks-PBS buffer (pH 7.4) and resuspended in an amikacin sulphate solution in Hanks-PBS buffer to obtain a cell suspension of 70% hematocrit and a 32 mM amikacin concentration. A 1-ml aliquot of the cell suspension was placed in the dialysis bag and incubated against a hypotonic buffer (15 mM NaH_2PO_4 $2H_2O$, 15 mM $NaHCO_3$, 20 mM glucose, 2 mM ATP, 3 mM reduced glutathione, 5 mM NaCl, 90 mOsm/Kg, pH 7.4) for 45 minutes at 4º C. Then, the dialysis bag was transferred to the resealing buffer (250 mM NaCl, 12.5 mM glucose, 12.5 mM sodium pyruvate, 12.5 mM inosine, 12.5 mM NaH_2PO_4 $2H_2O$, 0.63 mM adenine, 500 mOsm/Kg, pH 7.4) for 15 minutes at 37º C. Finally, drug-loaded erythrocytes were washed twice in Hanks-PBS buffer to remove unencapsulated drug.

2.2. *In vitro* uptake of amikacin by peritoneal macrophages

Peritoneal macrophages were obtained from Wistar rats treated with intraperitoneal 3% thioglycollate (Álvarez et al., 1998). Four days later, the macrophages were withdrawn by peritoneal lavage with PBS, pH 7.4, and washed. Cells were resuspended with RPMI 1640 culture medium supplemented with 10% foetal bovine serum, 4 mM L-glutamine and 1% ampicillin, and counted in a Neubauer chamber using Trypan blue to ensure cell viability. Following this, macrophages were resuspended in culture medium at a final concentration of $4x10^6$ cells/ml. Aliquots of the cell suspension were placed on microwell plates (1ml/well)

and incubated for 2 hours at 37 ºC in a humidified air/CO_2 (95/5%) atmosphere to obtain a cell monolayer. Non-adherent cells were removed by washing the wells with PBS. One ml of amikacin-loaded erythrocytes, brought up to 50% hematocrit in the culture medium, or 1 ml of 0.5 mg/ml amikacin sulphate solution were added to each well, and the plates were maintained for 30, 60, 90 or 120 minutes under the same conditions. Six replicates of each experiment were carried out. Then, the supernatant was removed by aspiration and the wells were washed with PBS. Adhered but non-ingested erythrocytes were lysed with 2 ml of 150 mM NH_4Cl for 3 minutes at room temperature. Finally, the macrophage monolayer was removed with 1 ml of 0.025% Triton X-100 in 1M NaOH.

2.3. *In vivo* amikacin distribution

For *in vivo* assays, 56 male Wistar rats with a mean weight of 256 $\pm$ 39 g were used. The animals were treated intraperitoneally with 3% thioglycollate. Four days later, they were injected intraperitoneally with 1 ml of amikacin-loaded erythrocytes, at a mean dose of 2.82 $\pm$ 1.06 mg/Kg, or with an amikacin solution at a dose of 7.5 mg/Kg, as a control group.

At different times after amikacin administration (3, 6, 12, 24 hours), the animals were killed and samples of peritoneal fluid, plasma, and tissues (liver, spleen, lung and kidney) were taken. Macrophages were isolated by peritoneal lavage and washed as described in the previous section.

2.4. Determination of amikacin concentrations

The amikacin concentrations in the different samples were determined with a high performance liquid chromatographic (HPLC) technique based on pre-column derivatization with O-phthaldialdehyde (OPA), using fluorescence detection (Santos et al., 2002). Tissue samples were previously homogenized in 1 ml/g of $6.7x10^{-2}$ M phosphate buffer, pH 7.4, and 0.25 mM Triton X-100 using a Pro 250 homogenizer.

Sample pretreatment was as follows. First, 100 µl of 20% trichloroacetic acid was added to 200 µl of sample for protein precipitation. The mixture was then

centrifuged for 5 minutes at 10900 rpm, and the supernatant was removed. 100 μl of 1M NaOH, 1 ml of phosphate buffer, pH 11, and 2 ml of methylene chloride were added to the supernatant and the mixture was centrifuged for 5 minutes at 3500 rpm. The aqueous phase was collected and mixed with 1 ml of OPA-reagent, and 500 mg of anhydrous sodium carbonate was added. To extract the amikacin-OPA complex, 500 μl of isopropanol was added and the mixture was centrifuged for 5 minutes at 3500 rpm. Finally, 20 μl of the alcoholic phase was injected into the chromatograph.

Chromatographic analysis was performed using a Shimadzu LC 10 AD chromatograph. The analytical column was a RP-18 LichroCart 5 cmx4mm I.D., 3 μm particle size. Samples were eluted using a mobile phase consisting of methanol/water (62/48%) with 2.2 g/l EDTA as an ion-pair reagent. Fluorescence detection was accomplished at excitation and emission wavelengths of 343 and 440 nm respectively. Flow rate was 1.5 ml/min. This technique has been validated in plasma and in liver homogenates according to FDA specifications, showing adequate linearity and a C.V. < 15% for accuracy and precision. The quantification limit was 0.1 μg/ml.

2.5. Data analysis

The macrophages, plasma, and tissue levels of amikacin administered as an aqueous solution or encapsulated in carrier erythrocytes were characterized with a model-independent analysis. Model-independent pharmacokinetic parameters in macrophages, plasma and tissues, such as the area under curve (AUC_{0-t}) and the mean residence time (MRT_{0-t}), were calculated using the trapezoidal rule. In addition, an *in vivo* tissue/plasma partition coefficient (P) was estimated using the tissue/plasma area under curve ratio ($(AUC_{0-t})_T/(AUC_{0-t})_P$). From a theoretical point of view, and for a non-eliminating blood flow-limited compartment, AUC ratios should be considered as a partition coefficient (P) calculated by an area method (Weiss, 1985). This ratio allows estimation of the targeting efficiency for each form of amikacin administration (control and loaded erythrocytes).

The relative uptake of amikacin in macrophages and tissues *in vivo* was estimated using the ratio between the partition coefficient using carrier erythrocytes (P_{eryt}) and the partition coefficient in the control group (P_{contr}), and the ratio between the area under the curve using carrier erythrocytes ($(AUC_{0-t})_{Teryt}$) and the area under the curve in the control group ($(AUC_{0-t})_{Tcontr}$).

Statistical comparison between the normalized plasma and tissue levels of amikacin in different groups of rats was carried out with non-parametric one-way analysis of variance using the SPSS 15.0 statistical software (Weitzman, 1999).

3. RESULTS

3.1. Amikacin uptake by macrophages *in vitro.*

Figure 1 shows the concentrations of amikacin in macrophages *in vitro* after the administration of the antibiotic as a solution and as loaded erythrocytes.

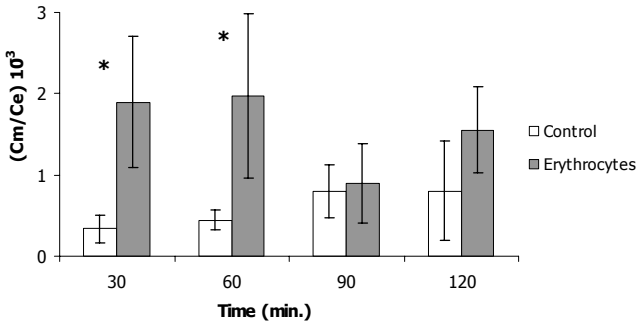

Figure 1. Amikacin concentrations, normalized by dose, in macrophages *in vitro* after administration as a solution (open bars) and as loaded erythrocytes (shaded bars) (*$p<0.05$).

A higher accumulation of the antibiotic was observed in peritoneal macrophages when the drug was administered in carrier erythrocytes. Statistically significant differences ($p<0.05$) at times 30 and 60 minutes were observed. No statistical differences at 90 and 120 minutes were found ($p>0.1$).

3.2. Amikacin uptake by macrophages and tissue distribution *in vivo.*

Figure 2 shows the amikacin levels in plasma, macrophages, and different tissues *in vivo*. In all plots, the amikacin levels were standardized by dose after the administration of the drug as a solution and in amikacin-loaded erythrocytes. Non-parametric statistical analysis of the concentrations revealed statistically significant differences in spleen ($p<0.001$), liver ($p<0.001$) and lung ($p<0.001$) and peritoneal macrophages ($p<0.001$).

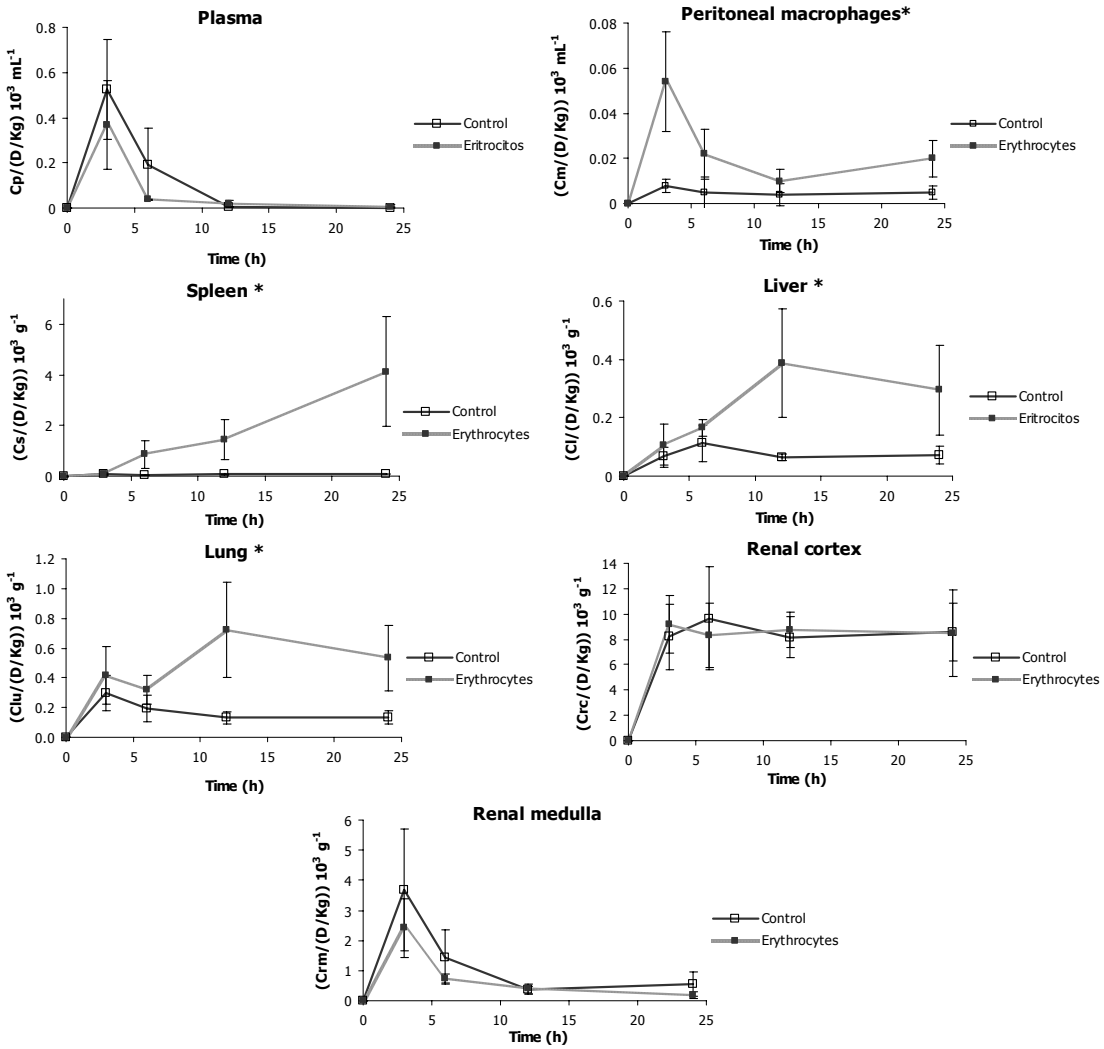

Figure 2. Amikacin concentrations, normalized by dose and weight, in plasma, macrophages and different tissues *in vivo* after i.p. administration of the antibiotic as a solution (□) and as loaded carrier erythrocytes (■) (* $p < 0.001$).

Table 1 shows the values of the area under the curve (AUC_{0-24}), the mean residence time (MRT_{0-24}), and the partition coefficients (P) of amikacin *in vivo* in the control group and in the carrier erythrocyte group. When amikacin-loaded carrier erythrocytes were administered, an increase in the area under the curve and/or the partition coefficient was observed in peritoneal macrophages, liver, spleen and lung in comparison with the control group.

Tissue	Control			Erythrocytes		
	$(AUC)_{0-24}$ **((μg/g)/(mg/Kg)) h**	**$(MRT)_{0-24}$** **(h)**	**P**	**$(AUC)_{0-24}$** **((μg/g)/(mg/Kg)) h**	**$(MRT)_{0-24}$** **(h)**	**P**
Plasma	2.11*	4.26		1.29*	5.39	
Macrophages	0.11*	12.13	0.05	0.46*	10.97	0.36
Liver	1.71	12.26	0.81	6.29	14.26	4.88
Spleen	1.43	13.30	0.68	41.96	18.47	32.52
Lung	3.77	11.11	1.79	12.38	13.56	9.59
Renal cortex	192.77	12.71	91.32	194.48	12.73	150.76
Renal medulla	22.74	8.24	10.78	14.50	7.65	11.24

* ((μg/ml)/(mg/Kg)) h

Table 1. Model-independent pharmacokinetic parameters of amikacin *in vivo* obtained in the control and carrier erythrocyte groups.

Figures 3 and 4 show the relative uptake of amikacin in peritoneal macrophages and tissues *in vivo* using the ratios between the areas under the curve and the partition coefficients obtained in carrier erythrocytes and in the control group. The relative uptake of amikacin obtained was: spleen > peritoneal macrophages > liver > lung > renal cortex > renal medulla.

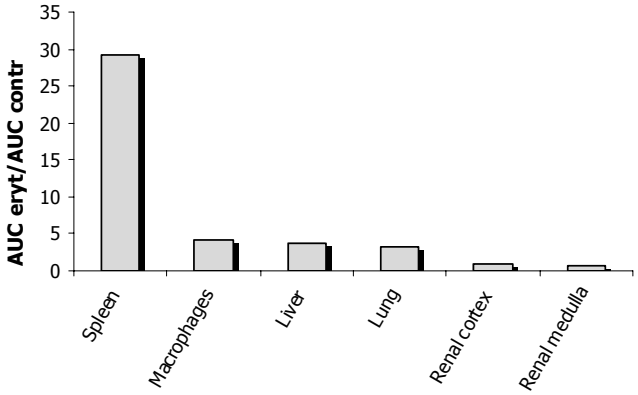

Figure 3. Relative uptake of amikacin in macrophages and tissues *in vivo* using the ratio between the area under the curve in tissue after administration of the antibiotic as carrier erythrocytes $(AUC_{0\text{-}24})_{eryt}$ and a drug solution $(AUC_{0\text{-}24})_{contr}$.

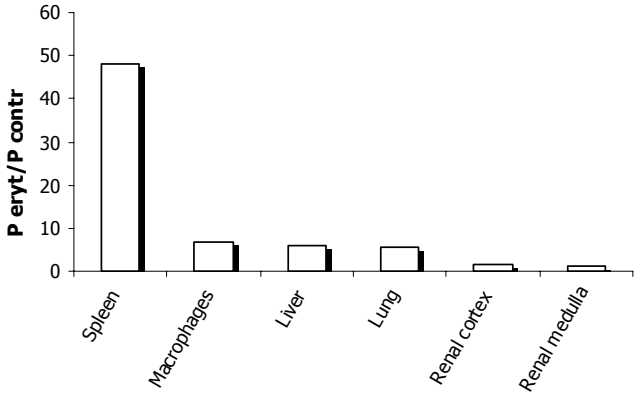

Figure 4. Relative uptake of amikacin in macrophages and tissues *in vivo* using the ratio between the partition coefficient(s) in tissue after administration of the antibiotic by means of carrier erythrocytes (P_{eryt}) and as a drug solution (P_{contr}).

4. DISCUSSION

The treatment of intracellular infections is a serious problem in classic antimicrobial therapies since it requires therapeutic antibiotic concentrations to be reached inside cells. This means that many antibiotics with proven efficacy against

intracellularly located pathogens *in vitro* show little or no antibacterial activity *in vivo*, considering that the antibiotic must be present at a sufficiently high concentration to be able to destroy the microorganism directly or to increase the antibacterial function of phagocytic cells (Prokesch, Hand, 1982). From a pharmacodynamic point of view, the intracellular concentration of the antibiotic is critical in drugs such as aminoglycosides. However, this kind of antibiotic has a limited capacity to penetrate cells. One strategy used to improve penetration consists of incorporating the antibiotic into delivery systems that are later ingested by phagocytic cells, selectively delivering the antibiotic to the target cell. Drug-containing erythrocytes are able to selectively direct antibiotics to the phagocytic cells of the RES and are potentially useful for the treatment of infections caused by intracellular microorganisms (Lanao, Sayalero, 2006).

Previous studies carried out with aminoglycoside antibiotics such as gentamicin or amikacin have shown that most of the encapsulated amikacin remains retained in erythrocytes for long periods of time and this allows an *in vivo* sustained release of the antibiotic from the erythrocytes, together with demonstrated RES targeting (Eichler et al., 1986; Gutiérrez et al., 2005; Gutiérrez et al., 2008).

The *in vitro* results concerning the accumulation of amikacin in peritoneal macrophage cell monolayers (Figure 1) show that amikacin-loaded erythrocytes are phagocytosed by macrophages, allowing a higher uptake of the antibiotic by this kind of phagocytic cell. However, cellular accumulation is not always predictive of the intracellular activity, since antibacterial activity is influenced by physicochemical factors, the metabolic state of the pathogen, and the effect of the antibiotic on the phagocytic capacity of the cell (Barcia-Macay et al., 2006; Lutwyche et al., 1998; Schiffelers et al., 2001; Lanao et al., 2007; Briones et al. 2008).

The plasma pharmacokinetics of amikacin after i.p. administration of antibiotic-loaded erythrocytes revealed a sustained release effect, with an increase in the plasma half-life of the antibiotic from 1.27 hours in the control group to 6.36 hours in the carrier erythrocyte group. As seen in Figures 3 and 4, the *in vivo* tissue

pharmacokinetics of amikacin revealed a greater accumulation of the antibiotic in the spleen, peritoneal macrophages and liver, and moderate changes in the pharmacokinetics in lung, renal cortex and medulla (Table 1). The greater accumulation of the antibiotic in peritoneal macrophages observed *in vivo* when carrier erythrocytes were administered is in agreement with the results obtained *in vitro* using macrophage cell monolayers.

Moreover, an important increase in amikacin levels in spleen and liver was observed, as seen in figure 2. A previous study examining the pharmacokinetics of amikacin in rats using carrier erythrocytes administered through the i.v. route also revealed changes in the pharmacokinetics of the antibiotic incorporated into this biological delivery system. Such changes involved a higher half-life and mean retention time and a higher accumulation of the antibiotic in liver and spleen (Gutiérrez et al., 2008), that are two important organs of the immune system. The liver contains many phagocytic cells, which capture bacteria from the blood when it passes through this organ. The spleen also contains phagocytic cells, both lymphocytes and monocytes. The increased levels of amikacin observed in the livers and the spleens of the animals treated with amikacin-loaded erythrocytes are correlated with the higher accumulation of amikacin in peritoneal macrophages both *in vitro* and *in vivo*.

In conclusion, amikacin-loaded erythrocytes increase the uptake of this antibiotic by phagocytic cells such as macrophages and may potentially be used as a delivery system to improve the efficacy of aminoglycoside antibiotics for the treatment of intracellular infections.

Acknowledgements

This work was supported by the Spanish National I+D+i Plan (Projects: PI060152 and SAF2001-0740).

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CAPÍTULO 5

Study of the factors influencing the encapsulation of zidovudine in rat erythrocytes

Enviado para su publicación a *International Journal of Pharmaceutics*

Study of the factors influencing the encapsulation of zidovudine in rat erythrocytes

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Abstract

Antiretroviral-loaded erythrocytes offer a promising therapy against HIV owing to their potential to deliver this kind of drugs to macrophages and reticulo-endothelial (RES) tissues. The aim of the present work was to develop and optimize a hypotonic dialysis method for the encapsulation of the antiretroviral Zidovudine (AZT) in rat erythrocytes. The influence of several factors in the encapsulation was also evaluated. Variables such as the initial AZT concentration, the dialysis time, and the dialysis bag volume exhibited statistically significant differences in the encapsulation of the drug in erythrocytes. The amount of drug encapsulated was related to the different values of the variables by multiple linear regression. Osmotic fragility and haematological parameters were estimated as indicators of erythrocyte viability. No statistically significant differences in the osmotic fragility profiles of the control and carrier erythrocytes were observed, and this parameter was also independent of the dialysis concentration of AZT, the hypo-osmotic dialysis time, and the dialysis/buffer volume ratio. The *in vitro* release of AZT from carrier erythrocytes pointed to a fast release of the drug; however, around 30% of the drug remained encapsulated for a prolonged period of time. Pre-dialysis diamide treatment did not have a significant effect on the encapsulation and release of AZT in erythrocytes.

Keywords: Zidovudine, carrier erythrocytes, hypotonic dialysis.

1. INTRODUCTION

Owing to the broad range of drugs available to treat HIV infection, the HIV-associated disease -acquired immunodeficiency syndrome (AIDS)- has been reduced to a chronic infection, at least in most developed countries. Nevertheless, highly active antiretroviral therapy (HAART) still has important limitations, such as the high cost of the treatments, the adverse effects of certain antiretroviral drugs, drug resistance, drug interactions, non-compliance problems, etc. (Kalkut, 2005; Lanao et al., 2007). In recent years, the struggle against HIV/AIDS has boosted the development of different therapeutic strategies, including the use of drug delivery systems to overcome these drawbacks.

Erythrocytes are potential biocompatible vectors for different bioactive substances, such as drugs, enzymes and other macromolecules. They have properties which make them suitable as drug carriers, such as their ability to provide a controlled-release effect or to target drugs to the reticulo-endothelial system, avoiding adverse effects in other organs, due to their rapid clearance from the blood by the monocyte-macrophage system. In comparison with other carrier systems, they have the advantage of higher biocompatibility, especially when autologous erythrocytes are used (Hamidi and Tajerzadeh, 2003; Gutiérrez et al., 2004b; Hamidi et al., 2007a; Hamidi et al., 2007b; Hamidi et al., 2007c; Briones et al., 2008; Patel et al., 2008).

Antiretroviral drugs show insufficient macrophage penetration capacities and low bioavailability, giving rise to the appearance of resistance and toxic effects due to their accumulation in other organs. The monocyte-macrophage system is the first to be infected by HIV. This supports the intracellular replication of the virus and acts as a reservoir, which favours the dissemination of the infection and protects the virus against antiretroviral treatment. The specific delivery of these agents into macrophages by means of drug delivery systems is therefore of huge therapeutic interest (Hu et al., 2000; Fraternale et al., 2002; Lanao et al., 2007; Briones et al., 2008).

The hypo-osmotic dialysis procedure is the method most frequently used for erythrocyte drug encapsulation because of its simplicity and relatively high yield of encapsulation (DeLoach and Ihler, 1977; Dale, 1987; DeLoach, 1987; Álvarez et al., 1998; Bax et al., 1999; Bax et al., 2000a,b; Gutiérrez et al., 2004a,b). The aim of this work was to study and optimize the factors that influence the encapsulation of zidovudine in rat erythrocytes, using a hypo-osmotic dialysis method.

2. MATERIAL AND METHODS

2.1. Materials

Zidovudine was kindly supplied by GlaxoSmithKline. All other chemicals and solvents were of analytical grade and used without further purification.

2.2. Blood collection and erythrocyte preparation

The housing and experimental treatment of the animals was in accordance with current Spanish and European Union legislation and complied with the "Principles of Laboratory Animal Care". Fresh blood was obtained from male Wistar rats by retro-orbital puncture, using EDTA (1.5 mg/ml) as anticoagulant. Plasma was removed after centrifugation of the blood (600× g, 5 min, 4ºC). Packed erythrocytes were washed twice with isotonic Hanks-PBS buffer (pH 8).

2.3. Encapsulation of zidovudine in carrier erythrocytes

The encapsulation of AZT in rat erythrocytes was accomplished using a hypotonic dialysis method (Eichler et al., 1986; Sanz et al., 1999; Gutiérrez et al., 2005). Packed erythrocytes were resuspended in an AZT solution in Hanks-PBS buffer to obtain a cell suspension of 70% hematocrit. The cell suspension was placed in the dialysis bag (Medicell, molecular size cut-off, 12-14 Kda.) and incubated against 50 ml of a hypotonic buffer (15 mM NaH_2PO_4 $2H_2O$, 15 mM $NaHCO_3$, 20 mM glucose, 2 mM ATP, 3 mM reduced glutathione, 5 mM NaCl, 90 mOsm/Kg, pH 8) at 4ºC. Then, the dialysis bag was transferred to 20 ml of the

resealing buffer (250 mM NaCl, 12.5 mM glucose, 12.5 mM sodium pyruvate, 12.5 mM inosine, 12.5 mM NaH_2PO_4 $2H_2O$, 0.63 mM adenine, 500 mOsm/Kg, pH 8) for 15 minutes at 37ºC. Finally, drug-loaded erythrocytes were washed twice in Hanks-PBS buffer to remove unencapsulated drug. Different conditions were tested in order to optimize the encapsulation method. The conditions assayed were: 1) Initial AZT concentrations in the cell suspension of 1, 2, 3, 6 and 10 mg/ml for a dialysis time of 45 min and a bag/buffer ratio of 1:50 ml, 2) Dialysis times of 45, 60 and 90 minutes for an initial AZT concentration of 10 mg/ml and a bag/buffer ratio of 1:50 ml, 3) Dialysis bag/hypotonic buffer ratios, using dialysis bag volumes of 1, 1.5 and 2 ml, against a fixed hypotonic buffer volume of 50 ml, for an initial AZT concentration of 10 mg/ml and a dialysis time of 45 min. Eight replicates of each experiment were carried out. In addition, the influence of diamide treatment on AZT encapsulation in erythrocytes was studied. To this end, washed erythrocytes were resuspended in 2 mM diamide in Tris/ClH buffer (40 mM Tris/ClH, 5 mM KCl, 116 mM NaCl, 0.2 mM $MgCl_2$, 5 mM glucose, pH 7.4) (Jordán et al. 2001; Lotero et al., 2001), and incubated at 37ºC for 1 hour. Then, cells were washed twice with the same buffer and hypotonic dialysis was carried out as described above.

2.4. Scanning electron microscopy (SEM)

In order to evaluate the existence of morphological changes in erythrocytes after the loading process, control and zidovudine-loaded ghost erythrocytes were prepared for scanning electron microscopy (SEM) as follows. Briefly, samples were incubated with poly-L-lysine for 1 hour and then fixed with 25% glutaraldehyde steam overnight. Samples were washed with phosphate buffer and dehydrated using a concentration gradient of acetone from 30 to 100%, and finally dry acetone. Then, critical point drying in liquid carbon dioxide and metallization with gold particles was carried out. The samples thus prepared were analyzed using a Zeiss DSM 940 electron microscope.

2.5. Measurement of haematological parameters

Haematological parameters were determined in an ADVIA TM 120 Hematology System analyzer. The parameters evaluated were haematocrit (HCT), released haemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).

2.6. Osmotic fragility

Osmotic fragility was determined with Dacie´s method (Dacie and Vaughan, 1938). Twenty-microlitre aliquots of carrier erythrocytes were incubated in 0.5 ml of an NaCl solution with a concentration ranging from 0 to 0.9% (w/v) for 30 minutes at room temperature, and then centrifuged. Haemoglobin release into supernatant was determined by spectrophotometry (λ=418 nm).

The amount of haemoglobin released was expressed as a percentage of the maximum haemolysis, which was determined as the amount of haemoglobin released into distilled water.

The osmotic fragility index was calculated for control and carrier erythrocytes as the NaCl concentration (% w/v) required to obtain 50% of haemolysis. Osmotic fragility was determined in triplicate for the different experimental conditions of this study.

2.7. *In vitro* release

The *in vitro* release of AZT from carrier erythrocytes was studied in erythrocytes prepared with 10 mg/ml as the initial AZT concentration, a dialysis time of 45 min, and a dialysis bag volume of 2 ml, with and without diamide treatment.

For these studies, carrier erythrocytes were resuspended in autologous plasma at a final haematocrit of 30%, separated into ten aliquots, and incubated at 37ºC with gentle stirring. At different times, aliquots were removed and centrifuged at 10,900 r.p.m. for 10 minutes. Sampling times were 5, 10, 15, 30 minutes, and 1,

2, 4, 8, 12, 24, and 48 hours. The amount of AZT released into the supernatant was determined by an HPLC technique, as described below.

Five replicates of this assay were carried out.

2.8. Quantification of encapsulated zidovudine

AZT concentrations in loaded erythrocytes were determined by a validated reverse-phase HPLC technique with UV detection (λ=265 nm). Chromatographic analysis was performed with a Shimadzu LC 10 AD chromatograph. The analytical column used was a RP-18 LichroCart 5 cmx4 mm I.D., 3 μm particle size. The mobile phase was 25 mM KH_2PO_4 pH=7/ acetonitrile (91:9%) at a flow rate of 1 ml/min. The retention time of AZT was 3.6 minutes. Sample pretreatment was performed by protein precipitation with perchloric acid. A 25-μl aliquot was diluted with 75 μl of saline, and 5 μl of 60% perchloric acid was added. The mixture was vortexed and centrifuged at 10,900 r.p.m. for 5 minutes. Then, 20 μl of the supernatant was injected into the chromatograph. The technique was validated according to FDA specifications (Guidance for Industry: Bioanalytical Method Validation, FDA, 2001). The method showed linearity in the concentration range used. Precision and accuracy had a C.V. of less than 6%.

2.9. Data analysis

Statistical analysis of data corresponding to AZT encapsulation, osmotic fragility and the haematological parameters was performed using SPSS 15.0 statistical software (Weitzman, 1999).

Analysis of variance (ANOVA) was performed, with dialysis time, the initial AZT concentration and dialysis bag volume as the three independent variables. In addition multilinear regression analysis was carried out. To assess the reliability of the parameters estimated, the estimated standard error, the 95% confidence limits, and the two-tail *p* value for a *t* test were evaluated.

Statistical comparison of the haematological parameters and osmotic fragility among the different conditions assayed were performed using non-parametric analysis.

Multivariate hierarchical cluster analysis with haematological parameters was performed. The haematological parameters evaluated were HCT, HGB, MCV, MCH and MCHC.

3. RESULTS

Figure 1 shows the results of scanning electron microscopy, where no significant changes in the shape or size of the erythrocytes can be observed.

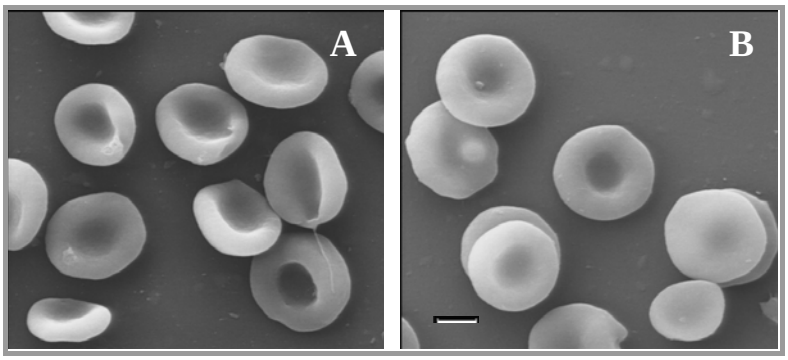

Figure 1. SEM micrographs of native (A) and AZT-loaded erythrocytes (B) (500 MAG) (bar 2 μm).

The results obtained demonstrate that higher initial concentrations of AZT, prolonged dialysis times, and a higher dialysis bag volume improve the encapsulation of AZT in erythrocytes. Analysis of variance (ANOVA) revealed statistically significant differences in the amount of AZT encapsulated for the different values of the factors tested.

Table 1 shows the results of the multilinear regression analysis. The amount of AZT encapsulated in erythrocytes was considered as the dependent variable and the initial AZT concentration, dialysis time and dialysis bag volume were considered

as the independent variables. The p values obtained indicated that all the independent variables considered were statistically significant ($p<0.001$).

Parameter	Value	95% confidence limits		Std. error	p	r
Constant	-909.510	-1241.060	-577.960	166.357	< 0.001	
Initial AZT conc. (mg/ml)	80.882	62.411	99.353	9.268	< 0.001	0.855
Dialysis time (min)	10.983	6.439	15.528	2.280	< 0.001	0.454
Dialysis bag volume (ml)	569.578	371.289	767.868	99.493	< 0.001	0.546

Table 1. Results of the multiple linear regression between the amount of AZT encapsulated in erythrocytes and the initial AZT concentration, the dialysis time, and the dialysis bag volume.

Figure 2 (A, B y C) shows the linear relationships obtained between the amount of AZT encapsulated in erythrocytes and the initial AZT concentration, dialysis time and the dialysis bag volume respectively, generated using the multilinear regression equation included in table 1.

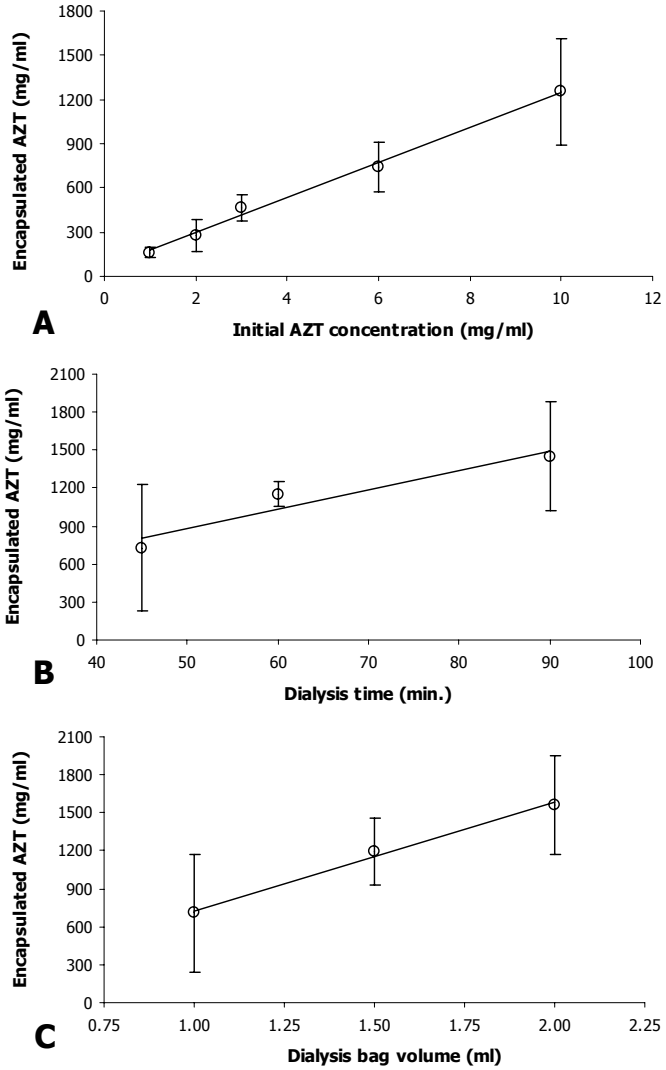

Figure 2. Linear regression between the amount of encapsulated AZT and the different factors assayed.

Table 2 shows the haematological parameters of loaded erythrocytes in comparison with native erythrocytes as a function of the initial AZT concentrations, dialysis times and dialysis bag volumes.

Initial AZT concentration (Dialysis time 45 min., dialysis volume 1 ml)					
Parameter	**HGB (g/dl)**	**HCT (%)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dl)**
Control	13.00 ± 0.92	37.50 ± 2.08	54.10 ± 4.42	18.73 ± 1.44	34.73 ± 2.80
1 mg/ml	13.70 ± 0.89	26.28 ± 6.77	70.18 ± 8.20	39.15 ± 13.18*	54.78 ± 14.14*
2 mg/ml	12.47 ± 1.33	24.77 ± 7.54	75.37 ± 0.42	39.50 ± 8.23*	52.40 ± 10.69
3 mg/ml	13.15 ± 0.66	30.85 ± 6.42	66.68 ± 9.85*	29.75 ± 9.47*	43.80 ± 7.70*
6 mg/ml	13.18 ± 0.46	29.45 ± 8.17	67.98 ± 6.45*	32.58 ± 0.75*	47.23 ± 12.11
10 mg/ml	13.68 ± 0.67	35.63 ± 2.49	62.03 ± 4.34	23.88 ± 2.35*	38.48 ± 2.08*
Dialysis time (Initial AZT concentration 10 mg/ml, dialysis volume 1 ml)					
Parameter	**HGB (g/dl)**	**HCT (%)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dl)**
45 min.	13.68 ± 0.67	35.63 ± 2.49	62.03 ± 4.34	23.88 ± 2.35*	38.48 ± 2.08*
60 min.	12.90 ± 1.57	27.43 ± 8.24*	70.60 ± 6.40*	35.88 ± 12.48*	50.00 ± 13.85*
90 min.	14.10 ± 2.62	25.90 ± 6.54 *	76.2 ± 0.91*	42.78 ± 11.11*	56.23 ± 14.91*
Dialysis bag volume (Initial AZT concentration 10 mg/ml, dialysis time 45 min.)					
Parameter	**HGB (g/dl)**	**HCT (%)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dl)**
1 ml	13.68 ± 0.67	35.63 ± 2.49	62.03 ± 4.34*	23.88 ± 2.35*	38.48 ± 2.08*
1.5 ml	13.75 ± 0.41	35.27 ± 1.59	66.37 ± 8.06*	25.92 ± 3.18*	39.03 ± 1.23*
2 ml	13.78 ± 0.82	36.90 ± 0.66	61.50 ± 4.4*	23.05 ± 2.96*	37.35 ± 2.48

Table 2. Mean hematological parameters of control and loaded erythrocytes and statistical significance using different conditions of encapsulation (*$p<0.05$).

Figure 3 shows the results of the cluster analysis of the haematological parameters using Euclidean distance and square root transformation.

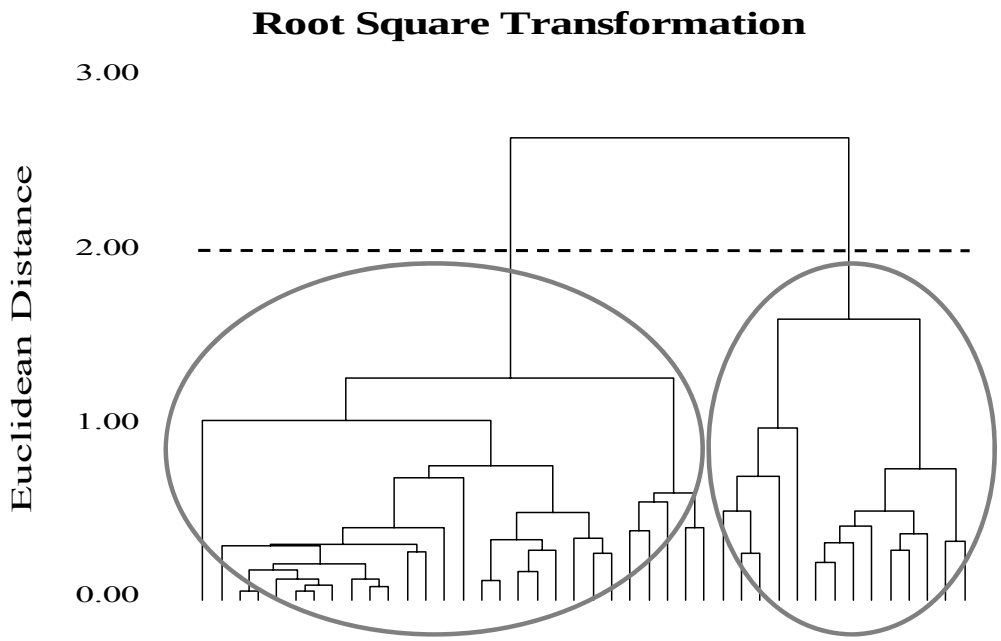

Figure 3. Cluster analysis of haematological parameters.

Figure 4 shows the osmotic fragility curves and table 5 depicts the osmotic fragility index of loaded erythrocytes in comparison with native erythrocytes as a function of the initial AZT concentrations (A), dialysis times (B) and dialysis bag volumes (C).

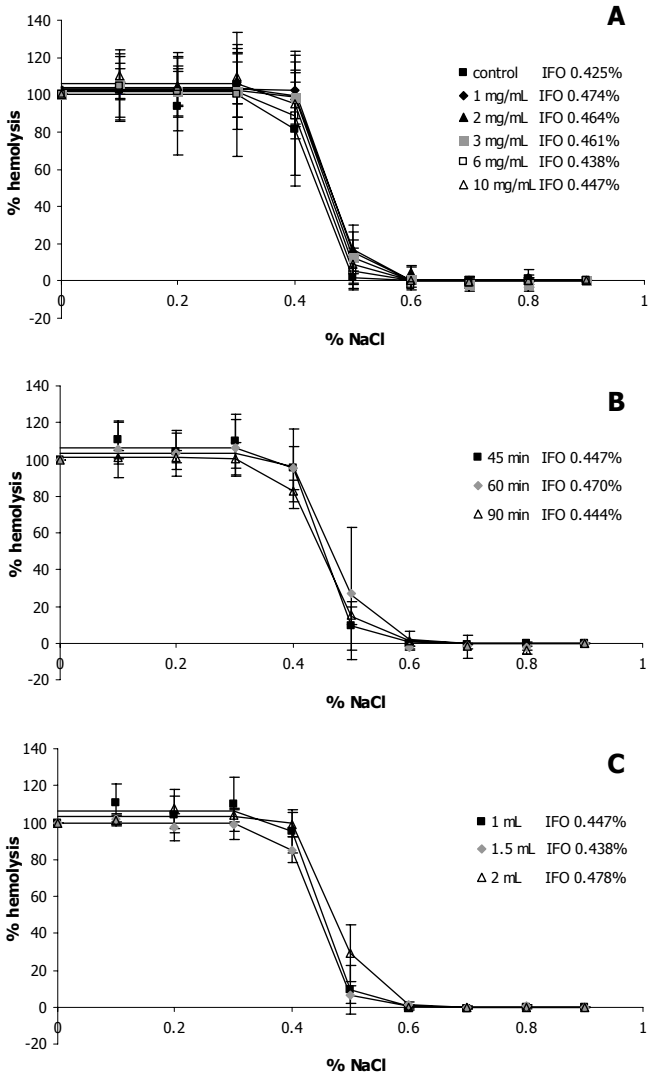

Figure 4. Osmotic fragility curves and osmotic fragility index (OFI) of control and carrier erythrocytes prepared using different initial AZT concentrations (A), dialysis times (B) and dialysis bag volumes (C).

Figure 5 shows the *in vitro* release curve of AZT from drug-loaded erythrocytes *versus* time. A rapid leakage of AZT from erythrocytes was observed. However, around 30% of the encapsulated drug remained inside the cell for

prolonged periods of time. Previous diamide treatment of loaded erythrocytes did not lead to significant differences in the amount of drug released ($p>0.05$).

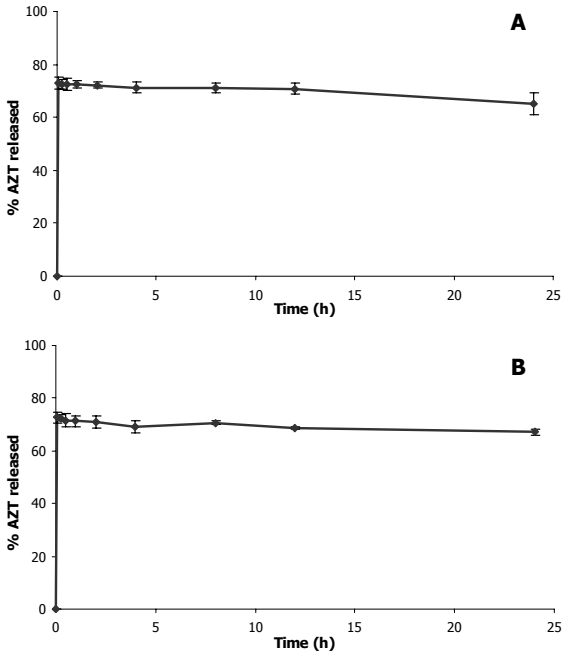

Figure 5. Release of zidovudine from carrier erythrocytes as a function of time: (A) AZT-erythrocytes, (B) AZT-erythrocytes with pre-dialysis diamide treatment.

4. DISCUSSION

The hypotonic dialysis method is widely used for the encapsulation of drugs and therapeutic substances in erythrocytes. This is because it is the technique that best preserves the biochemical and physiological characteristics of the erythrocytes resulting from that process. Despite its frequent use, however, the conditions used by different researchers to implement the method differ widely (Sanz et al., 1999; Gutierrez et al., 2004a,b, 2008; Murray et al., 2006).

In this study, an optimized method of zidovudine erythrocyte encapsulation aimed at achieving the highest encapsulated concentration without compromising the viability of the erythrocytes was developed. Considering the lipophilic character

of zidovudine, the uptake of the drug by carrier erythrocytes can be considered a passive process. According to the results shown in Table 1, a simultaneous dependence on the initial concentration of drug, dialysis time and dialysis bag volume in the yield of the encapsulation can be observed. An increase in the concentration of the drug in the dialysis bag will facilitate encapsulation into cells by means of a passive process (Lotero et al., 2003; Murray et al., 2006). Another essential feature in the process of drug encapsulation is the duration of the dialysis; this varies with the different substances to be encapsulated and ranges between 20 and 180 min, although dialysis times between 45 and 75 min are the most frequent (Gutiérrez et al, 2004a,b). In this study, a progressive increase in dialysis timed increased the amount of AZT encapsulated.

The influence of these factors -a high initial drug concentration and prolonged hypo-osmotic dialysis time- on encapsulation efficiency has been described previously for amikacin (Gutiérrez et al, 2008). However, the influence of the dialysis bag volume in the encapsulation of drugs has not yet been reported. As shown in Table 1, a progressive increase in dialysis bag volume from 1 to 2 ml incubated against the same volume of buffer improved the amount of drug encapsulated. This phenomenon may be related to an alteration of the drug gradient in the dialysis bag. Further studies with higher dialysis bag/hypotonic buffer volume ratios would be of interest to improve the yield of zidovudine encapsulation.

The haematological parameters showed statistically significant differences ($p<0.05$) with respect to the native erythrocyte controls for some of the different conditions assayed (table 2), especially when high dialysis times ($\geq$ 60 min) were tested. Therefore, the incubation time affected the viability of the erythrocytes, because statistically significant differences were observed in the haematological parameters such as HCT, MCV, MCH and MCHC ($p<0.05$) for an incubation time of 60 and 90 minutes with respect to erythrocytes incubated for only 45 minutes.

No statistical significant differences were found in the hematological parameters for any of the dialysis bag volumes used in this assay as compared with the initial volume (1 ml), eventhough statistically signifficant differences in some haematological parameters were found with respect to the control group. With multivariate hierarchical cluster analysis, and using a Euclidean distance of 2, the results revealed that the 5 variables led to the formation of two discrete clusters, as shown in Figure 5, confirming that extreme dialysis conditions, such as a dialysis time $\geq$ 60 minutes, elicit different haematologic behaviour.

As can be observed in Figure 3 for the different NaCl concentrations tested, no statistical differences in the release of haemoglobin with the initial concentration of the drug ($p>0.05$), the hypo-osmotic dialysis time ($p>0.05$) and the dialysis volume ($p>0.05$) were observed. The osmotic fragility index is slightly lower for native erythrocytes than the index estimated for AZT-loaded erythrocytes.

The kinetics of AZT release reveals a rapid leakage of drug from encapsulated erythrocytes; however, around 30% of the encapsulated drug remains entrapped in the cells for long periods of time. This type of behaviour allows the system to work as a drug reservoir, providing sustained release into the body and selectively directing the drug to the RES tissues (monocyte-macrophage system), such as the liver, spleen and bone marrow, which constitute the usual sites for the destruction of erythrocytes and are a reservoir for HIV viruses (Briones et al., 2008).

Diamide is an oxidizing agent that induces changes in the erythrocyte membrane. Such modifications can modulate recognition by macrophages, their survival in the circulation, and their removal by organs such as the liver and spleen (Lotero et al., 2001). Here, its influence in the encapsulating process and release of zidovudine was evaluated. Pre-dialysis diamide treatment failed to modify either the amount of drug encapsulated or the release kinetics of the drug.

According to the results obtained concerning encapsulation yield, haematologic parameters and osmotic fragility, the conditions finally selected for encapsulation were 10 mg/ml as the initial zidovudine concentration, and an

incubation time of 45 minutes and a dialysis bag/buffer volume ratio of 2:50 ml. The concentration of AZT encapsulated was 1.56 ± 0.39 mg/ml, and the encapsulation yield was 15.6%.

Acknowledgements

This work was supported by the projects I+D+i PI060152 and SA049A07.

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CAPÍTULO 6

Biodistribution of zidovudine encapsulated in carrier erythrocytes

Enviado para su publicación a *The AAPS Journal*

Biodistribution of zidovudine encapsulated in carrier erythrocytes

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Abstract

The monocyte-macrophage system plays an essential role in HIV infection as it can act as a reservoir for the virus, promoting the widespread distribution of the virus. The encapsulation of antiretroviral drugs in erythrocytes allows a higher accumulation in macrophages, improving their efficacy and avoiding side effects. The aim of this work was to study the biodistribution of zidovudine in rats using carrier erythrocytes as a delivery system. Zidovudine-loaded erythrocytes were obtained using a hypotonic dialysis method. The pharmacokinetic and tissue distribution of zidovudine were studied in two groups of rats previously induced with 3% thioglycolate and receiving intraperitoneal zidovudine in saline solution or encapsulated in carrier erythrocytes. Pharmacokinetic analysis was accomplished using model-independent methods. Zidovudine administration in the control group shows an accumulation of the drug in specific tissues such as kidney, lung and spleen. Administration of the antiretroviral drug using carrier erythrocytes produces significant changes in the plasma and tissue pharmacokinetics with an increase in the zidovudine plasma levels and in the tissue levels and terminal half-life in specific tissues like spleen, lung, kidney, peritoneal macrophages and monocytes from bone marrow. The administration of zidovudine carrier erythrocytes in rats leads to significant changes in the pharmacokinetics of the drug, a higher accumulation being observed in RES organs such as spleen and in phagocytic cells such as bone marrow monocytes and macrophages. This shows that loaded erythrocytes are potentially useful for the delivery of antiretroviral drugs in phagocytic cells located in the RES.

Key words: Zidovudine, carrier erythrocytes, pharmacokinetics, macrophages, biodistribution

INTRODUCTION

The treatment of HIV-associated disease, acquired immunodeficiency syndrome (AIDS), has been significantly improved by the introduction of highly active antiretroviral therapy (HAART), HIV becoming a chronic infection. Nevertheless, HAART still have important limitations such as the high cost of the treatments, the adverse effects, drug resistance, drug interactions, non-compliance problems, etc. (1, 2). Recently, many efforts have been made to search new therapeutic strategies in order to overcome these drawbacks, such as the use of drug delivery systems. A surge of publications about the development of carrier systems for antiretroviral drugs has emerged, mostly colloidal carriers such as liposomes (3-6) and micro/nanoparticles (6-11). However, biological carriers such as erythrocytes have advantages that make them more suitable for such purposes.

Erythrocytes are potential biocompatible vectors for different bioactive substances, such as drugs, enzymes and other macromolecules. They can provide a controlled release effect or target the drug to the reticulo-endothelial system, due to their clearance from the blood by the monocyte-macrophage system, avoiding adverse effects in other organs. Compared with other carrier systems, they show the advantage of a higher biocompatibility, especially when autologous erythrocytes are used (12-17).

HIV1 virus infects macrophages and T-helper linphocytes (CD4+), but the main characteristic of AIDS disease is the depletion of CD4+ cells. However, the monocyte-macrophage system, especially tissue macrophages, is able to resist the cytopathic effect of the virus, so that they can act as long-term reservoirs promoting the widespread distribution of the virus in all tissues. Hence, HIV is localized in compartments such as spleen, lungs, bone marrow, lymph nodes, or the central nervous system. Some of them are hardly accessible for most of the antiretroviral drugs, which cannot reach the therapeutic concentrations necessary for enough time in the infection site, giving rise to side effects because of the large doses

needed, and the development of drug resistance. The consequence is the failure of the treatment and the disease progression (6, 10, 18-20).

For these reasons, the specific delivery of these agents into macrophages by means of drug delivery systems is of huge therapeutic interest (1, 21-23). Carrier erythrocytes have been used previously as drug delivery systems for antiretroviral drugs showing interesting results for drug targeting to macrophages and RES tissues (23-25).

The aim of the present work was to study zidovudine (AZT) uptake by rat peritoneal macrophages *in vivo*, and the changes in its plasma pharmacokinetics and tissue distribution when it is administered in carrier erythrocytes through the intraperitoneal route.

MATERIAL AND METHODS

Zidovudine encapsulation in rat erythrocytes

The housing and experimental treatment of the animals were in accordance with current Spanish and European Union legislation and complied with the "Principles of Laboratory Animal Care".

The encapsulation of AZT in rat erythrocytes was accomplished using a hypotonic dialysis method (26). Briefly, fresh blood was obtained from male Wistar rats by retro-orbital puncture and centrifuged to remove plasma. Packed erythrocytes were washed twice with Hanks-PBS buffer (pH=8) and resuspended in an AZT solution in Hanks-PBS buffer to obtain a cell suspension of 70% hematocrit and a 37 mM (10 mg/mL) AZT concentration. A 2-mL aliquot of the cell suspension was placed in the dialysis bag and incubated against 50 mL of hypotonic buffer (15 mM NaH_2PO_4 $2H_2O$, 15 mM $NaHCO_3$, 20 mM glucose, 2 mM ATP, 3 mM reduced glutathione, 5 mM NaCl, 90 mOsm/Kg, pH 8) for 45 minutes at 4º C. Then, the dialysis bag was transferred to the resealing buffer (250 mM NaCl, 12.5 mM glucose, 12.5 mM sodium pyruvate, 12.5 mM inosine, 12.5 mM NaH_2PO_4 $2H_2O$, 0.63

mM adenine, 500 mOsm/Kg, pH 8) for 15 minutes at 37ºC. Finally, drug-loaded erythrocytes were washed twice in Hanks-PBS buffer to remove unencapsulated drug.

In vivo AZT distribution

For *in vivo* assays, 64 male Wistar rats with a mean weight of 249 ± 27 g were used. The animals were treated with an intraperitoneal injection of 3% thioglycollate. Four days later, they were injected intraperitoneally with 1mL of AZT-loaded erythrocytes at a dose of 12.71 ± 2.65 mg/Kg, or with an AZT solution at a dose of 8 mg/Kg, as a control group.

At different times after AZT administration (0.5, 1, 2, 3, 6, 12, 24, 48 hours), the animals were killed and samples of peritoneal fluid, plasma, and tissues (liver, spleen, lung, kidney, brain and bone marrow) were taken. Macrophages were isolated by peritoneal lavage and washed as described in the previous section. Bone marrow monocytes were flushed from femurs and washed with phosphate buffer saline (PBS) before cell counting, and resuspended in 1 mL PBS.

Determination of AZT concentrations

The zidovudine concentrations in the different samples were determined by ultra-performance liquid chromatographic coupled with positive-ion electrospray ionization tandem mass spectrometry (UPLC-ESI+MS/MS). Tissue samples were previously homogenized with 1 mL/g of $6.7x10^{-2}$ M phosphate buffer, pH 7.4, and 0.25 mM Triton X-100 using a Pro 250 homogenizer.

Samples pretreatment was made by protein precipitation with trichloroacetic acid. Briefly, 20 μL of 30% trichloroactic acid was added to 200 μL of sample. The mixture was vortexed and centrifuged at 10900 r.p.m. for 5 minutes. Supernatant was filtered using 0.2 μm filters, and then injected into the cromatograph. Chromatographic analysis was performed using a Waters Acquity UPLC system. The analytical column was a UPLC™ BEH C_{18} (50 mm x 2.1 mm i.d., 1.7 μm particle size)

at a temperature of 45ºC. Samples were eluted using a mobile phase consisting of 0.1% formic acid/acetonitrile (90/10%), at a flow rate of 0.5 mL/min. The retention time of zidovudine was 0.95 min. Detection was carried out with a Waters Acquity triple quadrupole mass spectrometer equipped with an ESI interface. Quantification was achieved with ESI source operating in positive mode, using multiple reaction-monitoring (MRM) of the transition *m/z* 268.20 $\rightarrow$ 127, with a scan time of 0.1 s per transition. The parameters of the spectrometer were: capillary 3 kV, cone energy 20 V, collision energy 15 V, source temperature 130ºC and desolvation temperature 450ºC. Nitrogen was used as the desolvation and cone gas at flow rates of 900 L/h and 50 L/h, respectively. Argon was used as the collision gas at a flow rate of 0.12 mL/min. All data collected were processed using MassLynx V4.1 software.

The technique was validated according to FDA specifications, showing adequate linearity and a C.V. < 15% for accuracy and precision (27).

Pharmacokinetic analysis

The tissue distribution of AZT administered as an aqueous solution or encapsulated in carrier erythrocytes was characterized by means of numerical deconvolution and conventional model-independent analysis.

Considering that drug transference between plasma and tissue is due only to passive diffusion, and that only the free drug fraction can be transferred, the tissue uptake rate of a drug in non-steady state situations can be calculated according to Eq.1 (28).

$$\frac{dX_T}{dt} = k\ (\ f_p C_p - f_T C_T\ ) \qquad (1)$$

where X_T is the total amount of drug in tissue; C_p and C_T are the plasma and tissue concentrations, respectively; f_p and f_T are the free fractions of drug in plasma and tissue, respectively; and k is a constant related to the intercompartmental clearance (Cl) or the regional blood flow (Q) in a flow-limited

distribution, depending on whether a compartmental or physiological approach is taken.

In practice, it is common to use drug concentrations instead of drug amounts, and hence Eq. 1 can be rewritten as follows:

$$\frac{dC_T}{dt} = \frac{k}{V_T} \left(f_p C_p - f_T C_T \right) \qquad (2)$$

where V_T is the distribution volume of tissue compartment.

Thus, the following simplified equation can be obtained:

$$\frac{dC_T}{dt} = K \left(PC_p - C_T \right) \qquad (3)$$

where P is the partition coefficient of the drug in a specific tissue defined as the tissue to plasma concentration ratio (C_T / C_p) at steady-state or the free fraction ratio (f_p / f_T) of the drug and K is a constant defined as follows:

$$K = \frac{k f_T}{V_T} \qquad (4)$$

Considering a linear and time-invariant system and integrating Eq. 3 using Laplace transformation, one obtains:

$$C_T(s) = \frac{PK}{(s+K)} C_p(s) \qquad (5)$$

where s is the Laplace operator.

Laplace antitransformation of Eq. 5 generates the following convolution integral:

$$C_T = \int_0^t PKe^{-K(t-\tau)} \, C_p(t) \, d\tau \qquad (6)$$

$$C_T = PKe^{-Kt} * C_p \qquad (7)$$

where "*" indicates the convolution operation.

Deconvolution "//" between C_T and C_p yields a unit disposition function (UDF) corrected by PK. The UDF represents the intratissue disposition of a unit amount of drug instantaneously injected into a tissue without recirculation (29).

$$UDF = C_T \,//\, C_p = PKe^{-Kt} \qquad (8)$$

where $PK = Cl/V_T$ when a compartmental approach is used and $PK = Q/V_T$ when a physiological approach is used. Equation 8 provides a parametric expression for the UDF.

Optimization of the UDF parameters was accomplished with the MULTI (FILT) program (30), which combines non-linear regression with numerical inversion of the Laplace transform. This program carries out the curve fitting of the tissues concentration-time data by non-linear regression using Laplace transformed equations. The UDF is convoluted with the input function characterized as a polyexponential equation. UDF parameters such as Cl_T/V_T and P were estimated by non-linear regression.

Considering that a significant fraction of the amount of AZT encapsulated in rat erythrocytes is rapidly released, a correction in the overall concentration of drug in tissues when AZT-carrier erythrocytes were administered was done. This correction in AZT plasma and tissue levels was performed using the polyexponential equation corresponding to the AZT plasma and tissue concentrations of the drug in the control group and the relative amount of drug encapsulated in erythrocytes established in *in vitro* studies previously conducted in our laboratory. These *in vitro* studies showed that 30% of the encapsulated drug remains in the erythrocytes for long periods of time (unpublished data).

In addition, conventional model-independent pharmacokinetic parameters in plasma and tissues like area under the curve ($AUC_{0-\infty}$), final phase elimination rate constant (λ_z), elimination half-life ($t_{½}$), mean residence time (MRT), mean transit

time (MTT) were calculated. MTT in tissues was calculated as the difference between the MRT in a specific tissue and plasma MRT (31,32).

Statistical analysis

The statistical comparison between normalized plasma and tissue levels of AZT in the two groups of rats was carried out with non-parametric analysis (Mann-Whitney test), using the SPSS 15.0 statistical software (33).

RESULTS

Figure 1 shows the mean zidovudine levels in plasma and different tissues standardized by the dose after the administration of zidovudine as a solution and in zidovudine-loaded erythrocytes. Non-parametric statistical analysis of the concentrations revealed statistically significant differences ($p<0.05$) for the shorter times (0.5 and 1 hour) for most of the tissues except kidney and bone marrow.

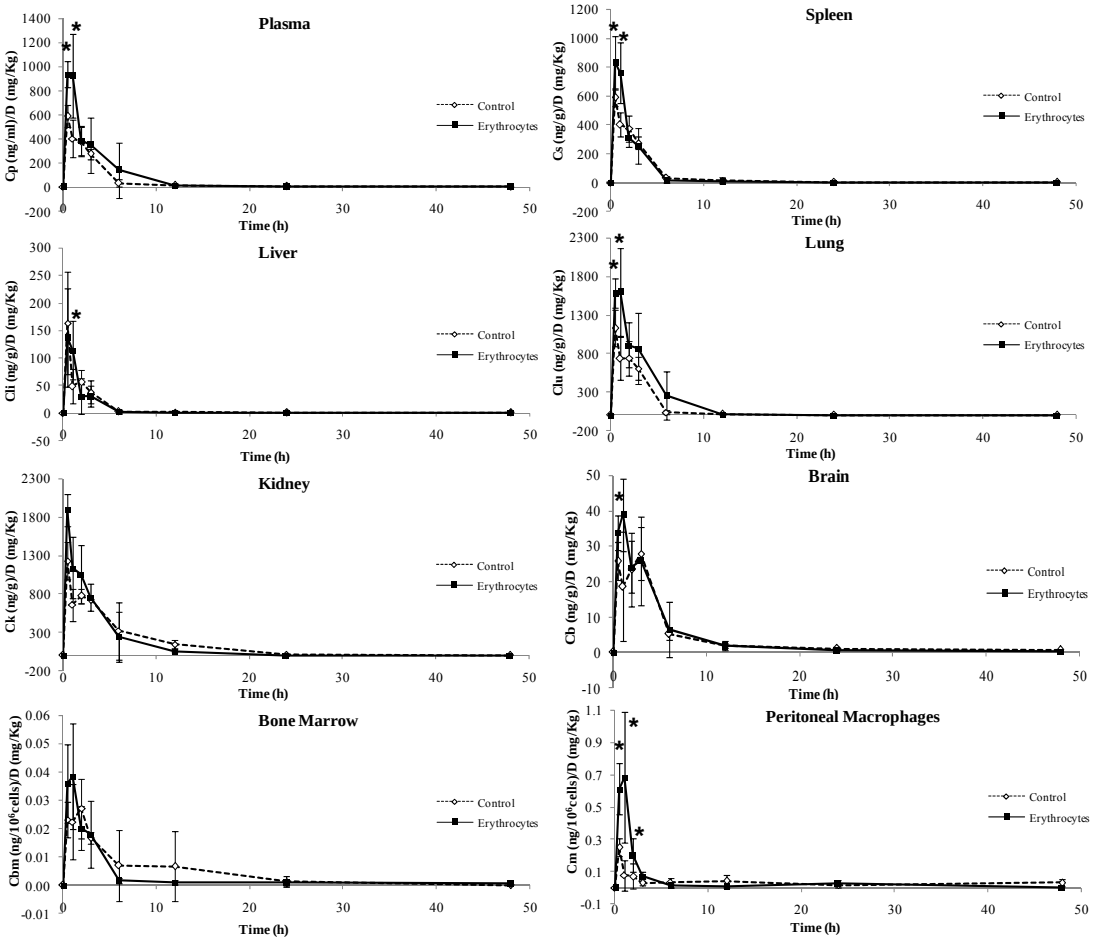

Figure 1. Mean zidovudine levels (± S.D.) in plasma and tissues standardized by the dose after the administration of zidovudine as a solution (control) and in zidovudine-loaded erythrocytes (* $p<0.05$).

Table 1 shows the estimated values of the parameters λ_z, $t_{½}$, $AUC_{0-\infty}$, MRT, MTT, CL_T/V_T and P in plasma and in different tissues after the administration of the drug in aqueous solution and incorporated in carrier erythrocytes.

Control group						
Tissue	**λz** (h^{-1})	**$t_{1/2}$** (h)	**$(AUC)_{0-\infty}$** ((ng/g)/(mg/Kg)) h	**MRT** (h)	**MTT** (h)	**P**
Plasma	0.108	6.44	1684.91	3.80	---	---
Spleen	0.134	5.17	1301.93	4.44	0.63	0.78
Liver	0.133	5.22	249.89	3.24	---	0.16
Lung	0.193	3.59	2937.20	2.75	---	1.75
Kidney	0.258	2.69	5414.81	4.84	1.04	1.96
Brain	0.026	26.45	180.23	18.37	14.57	7.20E-02
B. Marrow	0.100	6.91	0.19	8.46	4.65	5.00E-05
Macrophages	0.047	14.65	1.17	18.32	14.52	3.11E-02
Erythrocytes group						
Tissue	**λz** (h^{-1})	**$t_{1/2}$** (h)	**$(AUC)_{0-\infty}$** ((ng/g)/(mg/Kg)) h	**MRT** (h)	**MTT** (h)	**P**
Plasma	0.142	4.86	2750.99*	3.08	---	---
Spleen	0.093	7.44	1706.55	2.24	---	0.62
Liver	0.022	31.50	240.14	2.73	---	0.09
Lung	0.023	30.13	5335.09	3.17	0.09	2.02
Kidney	0.083	8.35	5459.46	3.41	0.34	1.98
Brain	0.032	21.79	174.24	9.54	6.46	0.05
B. Marrow	0.023	29.74	0.17**	15.21	12.14	6.18E-05
Macrophages	0.017	40.29	1.56**	20.17	17.09	5.67E-04

* ((ng/mL)/(mg/Kg)) h, ** ((ng/10^6cells)/(mg/Kg)) h

Table I. Model-independent pharmacokinetic parameters of zidovudine estimated in the control and carrier erythrocyte groups.

Figure 2 shows the simulation of the corrected concentrations of AZT in plasma and tissues corresponding to the administration of a unit dose of AZT encapsulated in erythrocytes and of the concentrations in the control group.

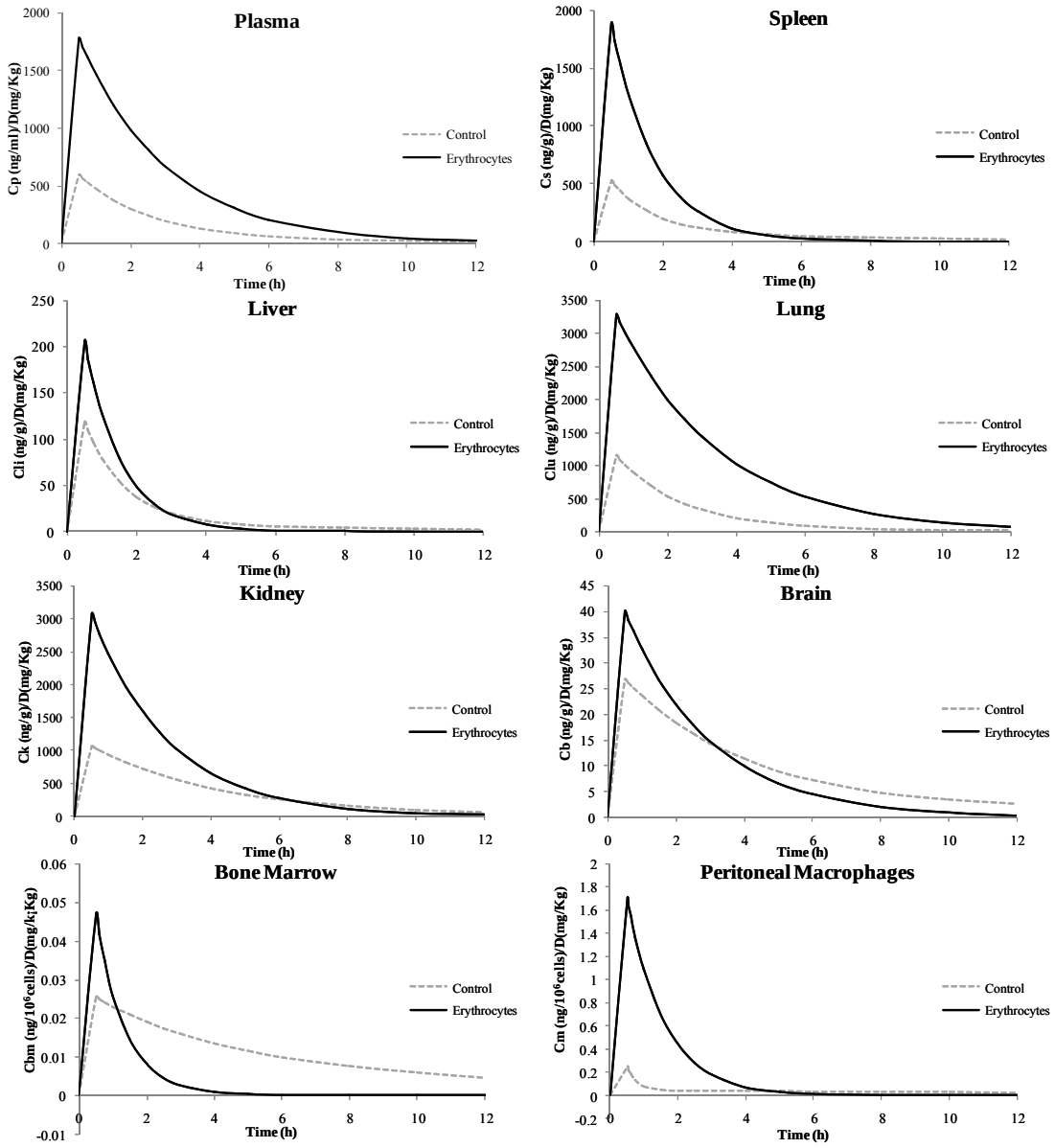

Figure 2. Simulation of the corrected concentrations of AZT in plasma and tissues corresponding to the administration of a unit dose of AZT encapsulated in erythrocytes comparatively with the concentrations obtained in the control group.

Figures 3 and 4 show comparatively the partition coefficient and the apparent half-life of AZT in tissues after the administration of the drug as a solution or as AZT loaded erythrocytes.

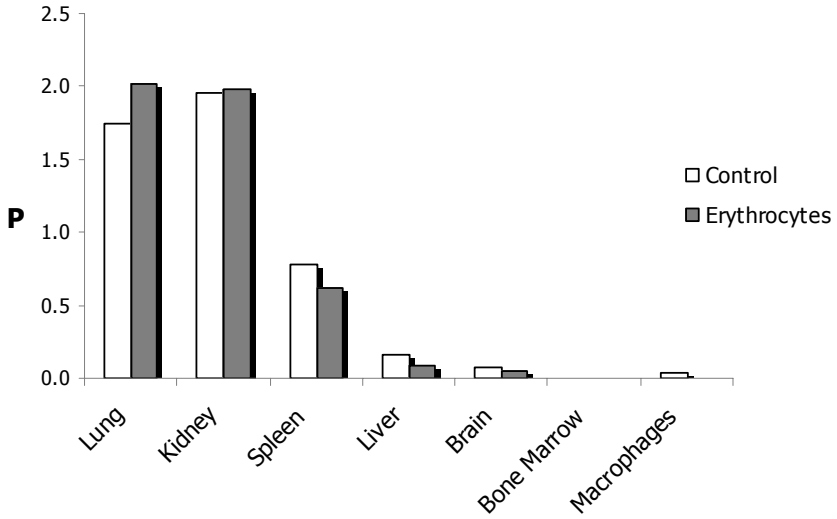

Figure 3. Partition coefficient of AZT in tissues after the administration of the drug as a solution or as AZT loaded erythrocytes.

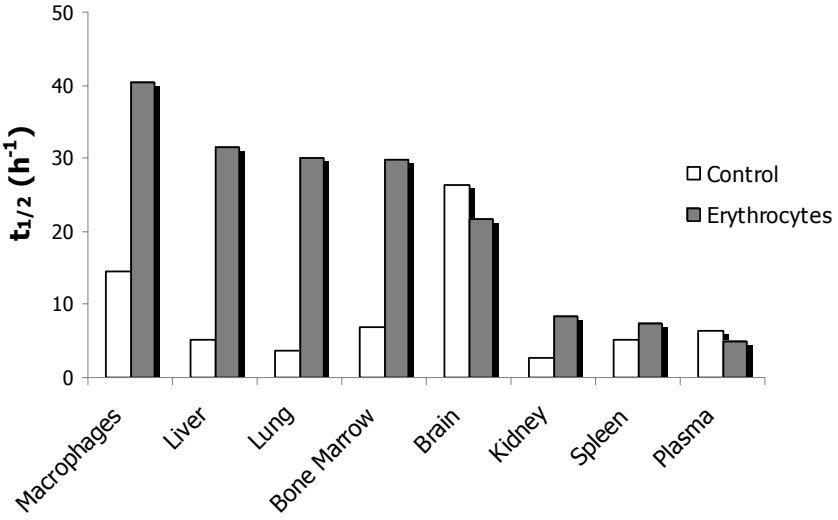

Figure 4. Apparent half-life of AZT in tissues after the administration of the drug as a solution or as AZT loaded erythrocytes.

DISCUSSION

Plasma pharmacokinetics of zidovudine is characterized by an intermediate half-life with values greater than 6 hours. In the group of animals receiving AZT as a drug solution, different tissue concentrations of AZT depending on the specific

organ or tissue studied were observed. As shown in the Figure 3 and Table 1, lung, kidney and spleen were the tissues with a higher partition coefficient. The lowest partition coefficient was observed in peritoneal macrophages and monocytes from bone marrow, although this partition coefficient was obtained using AZT concentrations normalized by dose and number of cells. However, the tissues with a lower partition coefficient like brain, peritoneal macrophages and monocytes show a prolonged apparent half-life and mean transit times as shown in Figure 4 and Table 1.

Viral infection of macrophages has been considered a critical step for the transmission of HIV-1. Considering that phagocytic cells like macrophages are the reservoir of HIV-1, tissues that are rich in resident macrophages like spleen, liver and lung are potential targets of AZT. The results obtained after administration of AZT as a solution by i.p. route in rats demonstrate a higher accumulation of AZT in lung and a moderate to low accumulation in spleen and liver.

The encapsulation of drugs, enzymes or other therapeutic substances in erythrocytes is able to give rise to a sustained release of the drug that affects the *in vivo* pharmacokinetic behaviour of the loaded drug in plasma and tissues (14, 34, 35).

Manipulated forms of erythrocytes as ghost erythrocytes containing drugs present morphological changes and increased osmotic fragility and are cleared from the circulation by the mononuclear phagocyte system through resident macrophages located in liver, spleen, lung and peritoneum.

The administration of AZT in loaded erythrocytes in rats significantly changed the pharmacokinetic behaviour of the antiretroviral drug in plasma, specific tissues and macrophages. As shown in Figure 1, the administration of AZT-loaded erythrocytes points to a higher accumulation of the drug in the target tissues like spleen and lung. In addition a higher accumulation of zidovudine in peritoneal macrophages and bone marrow monocytes was observed. The increase in the terminal half-life of zidovudine in tissues like spleen, liver or lung and especially in

peritoneal macrophages and monocytes suggests a longer stay of the drug in target tissues and phagocytic cells when this kind of drug is administered into carrier erythrocytes as shown in Figure 4.

This higher selectivity of zidovudine by target tissues when the drug is administered as carrier erythrocytes is highlighted in Figure 2 which shows the simulated levels of AZT in tissues for the control and carrier erythrocytes groups after the correction of the zidovudine tissue levels with the fraction of the dose released from carrier erythrocytes by a rapid leakage. This figure demonstrates the important modifications in the pharmacokinetic behaviour of zidovudine encapsulated in carrier erythrocytes in plasma and specific tissues like kidney, lung, spleen and peritoneal macrophages.

In spite of the modifications in zidovudine plasma and tissue pharmacokinetics when carrier erythrocytes are administered, the partition coefficient between tissue and plasma is not modified especially in well perfused tissues like spleen, liver or lung as shown in Figure 3. This may be attributed to the fact that the changes in the tissue pharmacokinetics of well perfused tissues are parallel to the modifications in plasma pharmacokinetics induced by the use of carrier erythrocytes.

CONCLUSION

AZT-loaded erythrocytes increase the drug levels in tissues like lung and spleen and in HIV-1 reservoirs such as peritoneal macrophages and monocytes. Therefore, this kind of delivery system may be used as a promising alternative to other delivery systems in antiretroviral therapy.

Acknowledgements

This work was supported by the projects I+D+i PI060152 and SA049A07.

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CAPÍTULO 7

Puesta a punto y validación de técnicas analíticas

En este capítulo se recoge la puesta a punto y validación de diferentes métodos analíticos para la cuantificación de amicacina y zidovudina en los distintos tipos de muestras biológicas obtenidos en los estudios realizados, como son eritrocitos, macrófagos, plasma y tejidos.

Concretamente, las técnicas que se han desarrollado son las siguientes:

Una técnica de HPLC (cromatografía líquida de alta resolución) con detección de fluorescencia para la cuantificación de amicacina.

Una técnica de HPLC con detección por ultravioleta para la cuantificación de zidovudina en eritrocitos de rata.

Una técnica de UPLC (cromatografía líquida ultra-rápida) con detección de masas (MS/MS) para la cuantificación de zidovudina en plasma y tejidos.

La validación de estas técnicas se ha realizado tomando como referencia las guía para la validación de técnicas bioanalíticas de la FDA (Guidance for Industry: Bioanalytical Method Validation, FDA, 2001).

El tratamiento de muestras, condiciones cromatográficas y resultados de la validación de cada una de estas técnicas se recogen a continuación.

1. CUANTIFICACIÓN DE AMICACINA MEDIANTE HPLC-FLUORESCENCIA.

Para la determinación de las concentraciones de amicacina en plasma, en macrófagos, en eritrocitos y en tejidos se utilizó una técnica de cromatografía líquida de alta eficacia (HPLC) de fase reversa con derivatización precolumna y detección por fluorescencia (Santos et al. 2002).

1.1. Tratamiento de las muestras.

Los tejidos fueron inicialmente tratados con 1 mL de Tritón X-100 de concentración 0,25 mM y 1 mL de tampón fosfato 6,7 10^{-2} M, pH 7,4 por gramo de tejido y homogeneizados con un homogenizador Pro 250.

Todas las muestras siguieron un proceso de acondicionamiento que consta de cuatro fases fundamentales:

Tratamiento con ácido tricloroacético que tiene como objetivo provocar la precipitación de las proteínas, así como la hidrólisis de organelas celulares y estructuras aniónicas a las que se encuentra unido el aminoglucósido, liberándose el fármaco de estas uniones en el sobrenadante.

Extracción del sobrenadante con cloruro de metileno, para eliminar componentes que puedan interferir en la reacción de derivatización posterior.

Reacción de derivatización con OPA (o-phtaldialdehído), ya que la amicacina no absorbe radiación ultravioleta ni es capaz de emitir radiación fluorescente. El OPA reacciona con aminas primarias y aminoácidos dando lugar en este caso a un complejo amicacina-OPA fluorescente. El reactivo derivatizante está compuesto por una disolución formada por 100 mL de tampón borato pH 10,4, 10 mL de metanol y 2 mL de mercaptoetanol, en la que se disuelve el OPA a una concentración de 4 mg por cada 5 mL (Santos et al. 1995).

Extracción del complejo amicacina-OPA con isopropanol.

El tampón fosfato a pH 11 y el NaOH se utilizan para ajustar el pH y el Na_2CO_3 para conseguir la separación de las fases acuosa y alcohólica.

El proceso completo de acondicionamiento, aparece recogido en la figura 6-1.

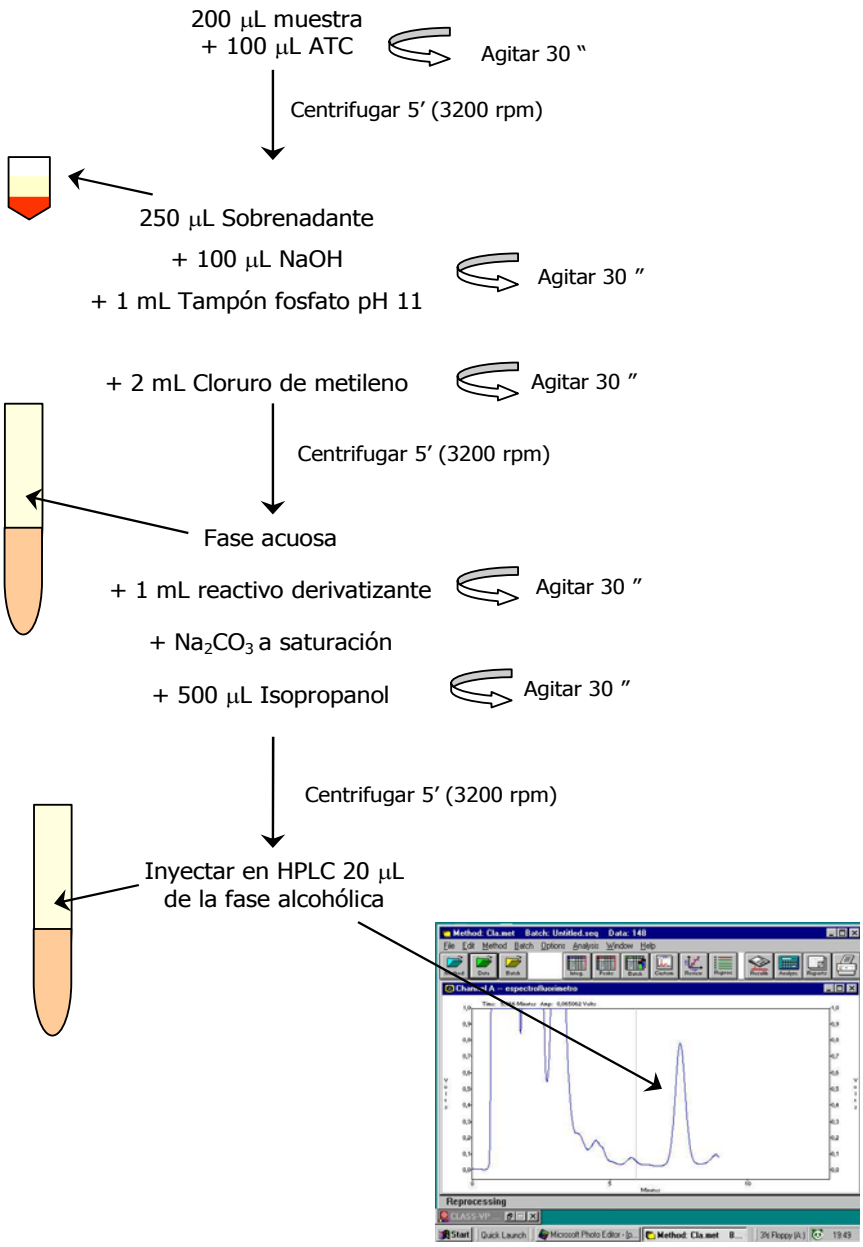

Figura 6-1. Tratamiento de muestras para la cuantificación de amicacina por HPLC.

1.2. Condiciones cromatográficas.

Se utilizó un cromatógrafo Shimadzu LC-10AD conectado a un detector de fluorescencia de longitud de onda variable Shimadzu 10AX y a un sistema de tratamiento de datos Shimadzu CLASS-VP. La detección se realizó a una longitud de onda de excitación de 343 nm, y de emisión de 460 nm.

El análisis cromatográfico de amicacina se llevó a cabo mediante una técnica de HPLC de par iónico, utilizando como contraión EDTA tripotásico.

La columna empleada fue una LiChroCart (Purospher® RP-18), con un tamaño de partícula de 3 μm, 5 cm de longitud y 4 mm de diámetro interno.

Se utilizó una fase móvil constituida por un 62% de metanol y 38% de agua destilada, con una concentración de EDTA tripotásico de 2,2 g/L de fase móvil. La velocidad de flujo de trabajo fue de 1,5 mL/min.

El proceso se llevó a cabo a una temperatura de 37ºC utilizando un horno externo Kontron.

El tiempo de retención fue de 12,5 minutos.

1.3. Validación de la técnica analítica.

La técnica fue validada en plasma y en homogenado de hígado. Se estudió la selectividad, linealidad, límite de cuantificación, exactitud y precisión.

1.3.1. Selectividad.

La selectividad del método se estudió analizando seis muestras de plasma y de hígado procedentes de seis ratas diferentes. En ningún caso se observaron picos que pudieran interferir con la amicacina (Figura 6-2).

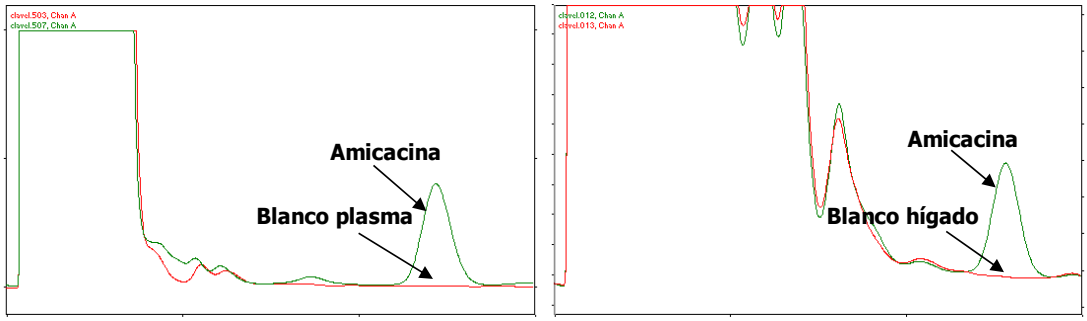

Figura 6-2. Cromatograma de un blanco y un patrón de amicacina en plasma (10 μg/mL) y en hígado (5 μg/mL).

1.3.2. Linealidad y límite de cuantificación.

La linealidad se estudió en dos intervalos de concentraciones en plasma, 0,1-2 μg/mL y 2-25 μg/mL, y en el intervalo 0,1-5 μg/mL en homogenado de hígado, utilizando seis concentraciones diferentes en cada una de las rectas. Para estudiar la linealidad se analizaron cinco replicados de cada recta. El área de los picos se relacionó con las concentraciones mediante una ecuación lineal del tipo:

$$y=a+bx$$

siendo *y* el área del pico cromatográfico, *x* la concentración de amicacina en la recta patrón, *b* la pendiente de la recta y *a* la ordenada en el origen. Se utilizó un método de regresión lineal de mínimos cuadrados utilizando $1/y^2$ como factor de ponderación, y se calcularon los coeficientes de correlación (r) y determinación (r^2).

Los resultados obtenidos se muestran en la siguiente tabla:

Recta	a	p(a)	b	p(b)	r	r^2
Plasma (0,1-2 μg/mL)	220207,9	<0,001	4921366,0	<0,001	0,997	0,995
Plasma (2-25 μg/mL)	244240,5	0,233	2071507,8	<0,001	0,992	0,984
Hígado (0,1-5 μg/mL)	-130384,9	<0,001	3989638,0	<0,001	0,997	0,994

Tabla 6-1. Parámetros obtenidos tras el análisis de regresión lineal ponderada.

En todos los casos la técnica fue lineal con coeficientes de correlación y determinación próximos a 1 y valores de la pendiente distintos de cero ($p < 0,05$). Para la recta en plasma 0,1-2 μg/mL y la recta en hígado la técnica fue proporcional ($p(b)<0,05$), pero no fue proporcional para la recta 2-25 μg/mL ($p(b)>0,05$).

Se definió como límite de cuantificación la concentración más baja de la recta de calibrado, 0,1 μg/mL en las dos matrices estudiadas, que se estimó con una buena exactitud y precisión (6 replicados C.V. < 15%).

1.3.3. Exactitud.

Para el estudio de la exactitud se analizaron cinco replicados de tres concentraciones (baja, media y alta) de cada recta de calibrado. Las concentraciones se calcularon utilizando la recta obtenida en el apartado anterior y se determinó el porcentaje de recuperación (($C_{exp}/C_{teórica}$) x100). Los resultados obtenidos se muestran en la Tabla 6-2. En todos los casos se obtuvieron C.V. del porcentaje de recuperación inferiores al 11%.

Recta	Conc. Teórica (μg/mL)	% Recuperación Media ± D.E.	C.V.
Plasma (0,1-2 μg/mL)	0,1	102,36 ± 8,45	8,26
	1	102,35 ± 3,92	3,83
	2	101,45 ± 6,07	5,98
Plasma (2-25 μg/mL)	2	98,71 ± 10,76	10,90
	10	104,43 ± 2,78	2,67
	25	95,81 ± 6,58	6,87
Hígado (0,1-5 μg/mL)	0,1	102,40 ± 8,91	8,70
	1	99,14 ± 3,38	3,41
	5	102,98 ± 3,26	3,16

Tabla 6-2. Resultados obtenidos en el estudio de la exactitud.

1.3.4. Precisión.

La precisión intradía (repetibilidad) se estudió analizando cinco replicados de tres concentraciones de cada recta en el mismo día. Para la precisión interdía

(reproducibilidad) se analizaron tres concentraciones por duplicado durante cuatro días. Los resultados obtenidos se recogen en las tablas 6-3 y 6-4. Los C.V. de las respuestas fueron en todos los casos inferiores al 14%.

Recta	Conc. Teórica (μg/mL)	Respuesta Media ± D.E.	C.V.
Plasma (0,1-2 μg/mL)	0,1	723964,60 ± 41604,00	5,75
	1	5257219,60 ± 193163,59	3,67
	2	10205691,60 ± 597347,24	5,85
Plasma (2-25 μg/mL)	2	4333642,20 ± 445822,10	10,29
	10	21876382,00 ± 576608,53	2,64
	25	49864372,80 ± 3407051,78	6,83
Hígado (0,1-5 μg/mL)	0,1	278150,40 ± 36373,94	13,08
	1	3824381,80 ± 134357,61	3,51
	5	20413866,00 ± 650516,56	3,19

Tabla 6-3. Resultados obtenidos en el estudio de la precisión intradía.

Recta	Conc. Teórica (μg/mL)	Respuesta Media ± D.E.	C.V.
Plasma (0,1-2 μg/mL)	0,1	622883,00 ± 70410,35	11,30
	1	5180034,38 ± 270133,17	5,21
	2	11820929,25 ± 966594,22	8,18
Plasma (2-25 μg/mL)	2	5498361,38 ± 629493,45	11,45
	10	24213574,50 ± 2158659,90	8,92
	25	58971639,50 ± 5372151,91	9,11
Hígado (0,1-5 μg/mL)	0,1	278653,50 ± 13474,51	4,84
	1	3913004,50 ± 47772,28	1,22
	5	21509006,50 ± 1166236,70	5,42

Tabla 6-4. Resultados obtenidos en el estudio de la precisión interdía.

2. CUANTIFICACIÓN DE ZIDOVUDINA MEDIANTE HPLC-UV.

Para la determinación de las concentraciones de zidovudina en eritrocitos se utilizó una técnica de cromatografía líquida de alta eficacia (HPLC) de fase reversa con detección de ultravioleta.

2.1. Tratamiento de las muestras.

El acondicionamiento de las muestras se realizó mediante precipitación proteica con ácido perclórico. 25 μL de muestra se diluyeron con 75 μL de suero fisiológico, y se añadieron 5 μL de ácido perclórico al 60%. Las muestras se centrifugaron 5 minutos a 10900 r.p.m. y 20 μL del sobrenadante se inyectaron en el cromatógrafo.

2.2. Condiciones cromatográficas.

Se utilizó un cromatógrafo Shimadzu LC-10AD conectado a un detector de ultravioleta de longitud de onda variable Kontron y a un sistema de tratamiento de datos Shimadzu CLASS-VP. La detección se realizó a una longitud de onda de 265 nm.

La columna empleada fue una LiChroCart (Purospher® RP-18), con un tamaño de partícula de 3 μm, 5 cm de longitud y 4 mm de diámetro interno.

Se utilizó una fase móvil constituida por un 91% de tampón fosfato pH=7, y 9% de acetonitrilo. La velocidad de flujo de trabajo fue de 1 mL/min.

El proceso se llevó a cabo a una temperatura de 30ºC utilizando un horno externo Kontron.

El tiempo de retención fue de 3,6 minutos.

2.3. Validación de la técnica analítica.

La técnica fue validada en eritrocitos. Se estudió la selectividad, linealidad, exactitud y precisión.

2.3.1. Selectividad.

La selectividad del método se estudió analizando seis muestras de eritrocitos procedentes de seis ratas diferentes. En ningún caso se observaron picos que pudieran interferir con la zidovudina (Figura 6-3).

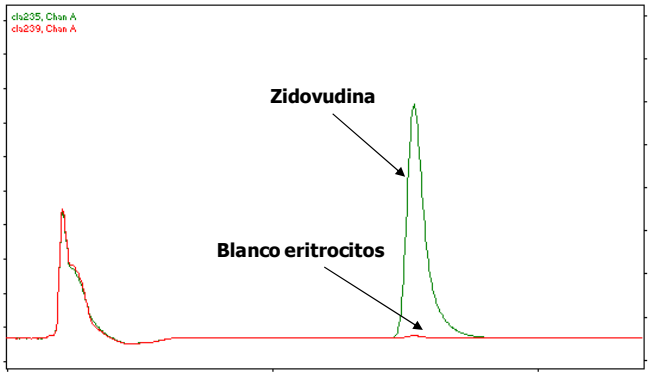

Figura 6-3. Cromatograma de un blanco y un patrón de zidovudina en eritrocitos (500 μg/mL).

2.3.2. Linealidad.

La linealidad se estudió en el intervalo de concentraciones 100-2000 μg/mL utilizando seis concentraciones diferentes. Para estudiar la linealidad se analizaron cinco replicados de la recta en el mismo día. Se utilizó un método de regresión lineal de mínimos cuadrados. El área de los picos se relacionó con las concentraciones mediante una ecuación lineal del tipo:

$$y=a+bx$$

siendo *y* el área del pico cromatográfico, *x* la concentración de zidovudina en la recta patrón, *b* la pendiente de la recta y *a* la ordenada en el origen, y se calcularon los coeficientes de correlación (r) y determinación (r^2).

Los resultados obtenidos en el análisis estadístico de la regresión se muestran en la siguiente tabla:

a	p(a)	b	p(b)	r	r^2
3954,98	0,0086	327,11	<0,001	0,999	0,999

Tabla 6-5. Parámetros obtenidos tras el análisis de regresión lineal.

La técnica fue lineal con coeficientes de correlación y determinación próximos a 1 y p(a)<0,05, y proporcional ya que p(b)<0,05.

2.3.3. Exactitud.

Para el estudio de la exactitud se analizaron cinco replicados de tres concentraciones (baja, media y alta) de la recta de calibrado. Las concentraciones se calcularon utilizando la recta obtenida en el apartado anterior y se determinó el porcentaje de recuperación (($C_{exp}/C_{teórica}$) x100). Los resultados obtenidos se muestran en la Tabla 6-6. El C.V. obtenido fue inferior al 4%.

Conc. Teórica (μg/mL)	% Recuperación Media ± D.E.	C.V.
100	104,04 ± 3,26	3,14
500	98,71 ± 1,82	1,84
2000	100,22 ± 1,06	1,06

Tabla 6-6. Resultados obtenidos en el estudio de la exactitud.

2.3.4. Precisión.

La precisión intradía (repetibilidad) se estudió analizando cinco replicados de tres concentraciones de cada recta en el mismo día. Para la precisión interdía (reproducibilidad) se analizaron tres concentraciones por duplicado durante cuatro días. Los resultados obtenidos se recogen en las tablas 6-7 y 6-8. Los C.V. fueron en todos los casos inferiores al 6%.

Conc. Teórica (μg/mL)	Respuesta Media ± D.E.	C.V.
100	37986,80 ± 1067,38	2,81
500	165391,40 ± 2970,15	1,80
2000	659584,60 ± 6965,57	1,06

Tabla 6-7. Resultados obtenidos en el estudio de la precisión intradía.

Conc. Teórica (μg/mL)	Respuesta Media ± D.E.	C.V.
100	35147,75 ± 2032,44	5,78
500	167214,88 ± 8564,17	5,12
2000	657845,63 ± 28601,28	4,35

Tabla 6-8. Resultados obtenidos en el estudio de la precisión interdía.

3. CUANTIFICACIÓN DE ZIDOVUDINA MEDIANTE UPLC-MS/MS.

Para la determinación de las concentraciones de zidovudina en plasma, en macrófagos y en tejidos se utilizó una técnica de UPLC (Ultra Performance Liquid Chromatography) de fase reversa con detección por espectrometría de masas (MS/MS) con ionización por electrospray (ESI).

La tecnología UPLC es de reciente introducción. Se basa en los principios de la cromatografía líquida para la separación cromatográfica, pero utiliza columnas con un tamaño de partícula inferior a 2 μm como fase estacionaria, y permite trabajar con presiones mayores. Ésto permite realizar un análisis más rápido, con una buena resolución cromatográfica y gran sensibilidad, y con un menor consumo de muestras (Liu et al. 2008, Dasandi et al. 2009).

La espectrometría de masas permite la separación e identificación de diferentes compuestos en función de la relación masa/carga (m/z). Para ello la muestra es ionizada por bombardeo con electrones que, en la modalidad MS/MS, van a romper la molécula, dando lugar a una fragmentación patrón que es

específica para cada sustancia. Esto permite identificar compuestos desconocidos, cuantificar compuestos conocidos, y aporta información sobre la estructura y propiedades químicas de la molécula. Presenta las ventajas de una sensibilidad mayor que otros métodos, y unos espectros sencillos y fácilmente interpretables. Entre los inconvenientes están el elevado coste de los equipos, y la deriva del instrumento que puede ser del orden del 5-10%/hora (Corral 2006), lo que conduce a una mayor variabilidad.

Por lo tanto, la utilización de un sistema UPLC acoplado a un tandem MS/MS va a proporcionar una mayor sensibilidad y especificidad en el análisis, lo que es de gran utilidad para la realización de estudios farmacocinéticos.

3.1. Tratamiento de las muestras.

Los tejidos fueron inicialmente tratados con 1 mL de Tritón X-100 de concentración 0,25 mM y 1 mL de tampón fosfato 6,7 10^{-2} M, pH 7,4 por gramo de tejido y homogeneizados con un homogenizador Pro 250.

El acondicionamiento de las muestras se hizo mediante precipitación proteica con ácido tricloroacético. A 200 μL de muestra se añadieron 20 μL de ácido tricloroacético al 30%. Las muestras se centrifugaron 5 minutos a 10900 r.p.m. y el sobrenadante se filtró a través de filtros de 0,2 μm (PTFE Membrane, 0,2 μm, Phenex™) antes de su inyección en el cromatógrafo. El volumen de inyección fue 10 μL para las concentraciones bajas y 1 μL para las concentraciones altas.

3.2. Condiciones cromatográficas.

Se utilizó un cromatógrafo Waters Acquity UPLC conectado a un detector de masas triple cuadrupolo y a un sistema de tratamiento de datos Waters MassLynx V4.1.

La columna empleada fue una UPLC BEH C_{18}, con un tamaño de partícula de 1,7 μm, 5 cm de longitud y 2,1 mm de diámetro interno, a una temperatura de 45 ºC.

Se utilizó una fase móvil constituida por un 90% de ácido fórmico al 0,1%, y 10% de acetonitrilo. La velocidad de flujo de trabajo fue de 0,5 mL/min.

La detección se realizó mediante MS/MS con ESI en modo de ionización positivo. La cuantificación se realizó utilizando monitorización de reacción múltiple (MRM) de la transición de m/z 268,20 → 127, con un tiempo de barrido de 0,1 seg. por transición.

Las condiciones del espectrómetro de masas fueron las siguientes: capilaridad 3 kV, energía de cono 20 V, energía de colisión 15 V, temperatura de fuente 130 ºC, temperatura de desolvatación 450 ºC. Se utilizó nitrógeno como gas de desolvatación y de cono con un flujo de 900 L/h y 50 L/h respectivamente. Como gas de colisión se utilizó argón a un flujo de 0,12 mL/min.

El tiempo de retención fue de 0,95 minutos.

3.3. Validación de la técnica analítica.

La técnica fue validada en plasma. Se estudió la selectividad, linealidad, límite de cuantificación, exactitud y precisión.

3.3.1. Selectividad.

La selectividad del método se estudió analizando seis muestras de plasma procedentes de seis ratas diferentes. En ningún caso se observaron picos que pudieran interferir con la zidovudina (Figura 6-4).

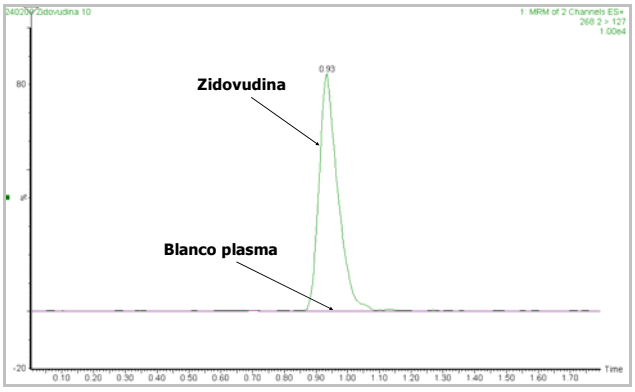

Figura 6-4. Cromatograma de un blanco y un patrón de zidovudina (25 ng/mL) en plasma.

En la figura 6.5 se muestran el espectro de masas para la zidovudina y los fragmentos obtenidos tras la ruptura de la molécula.

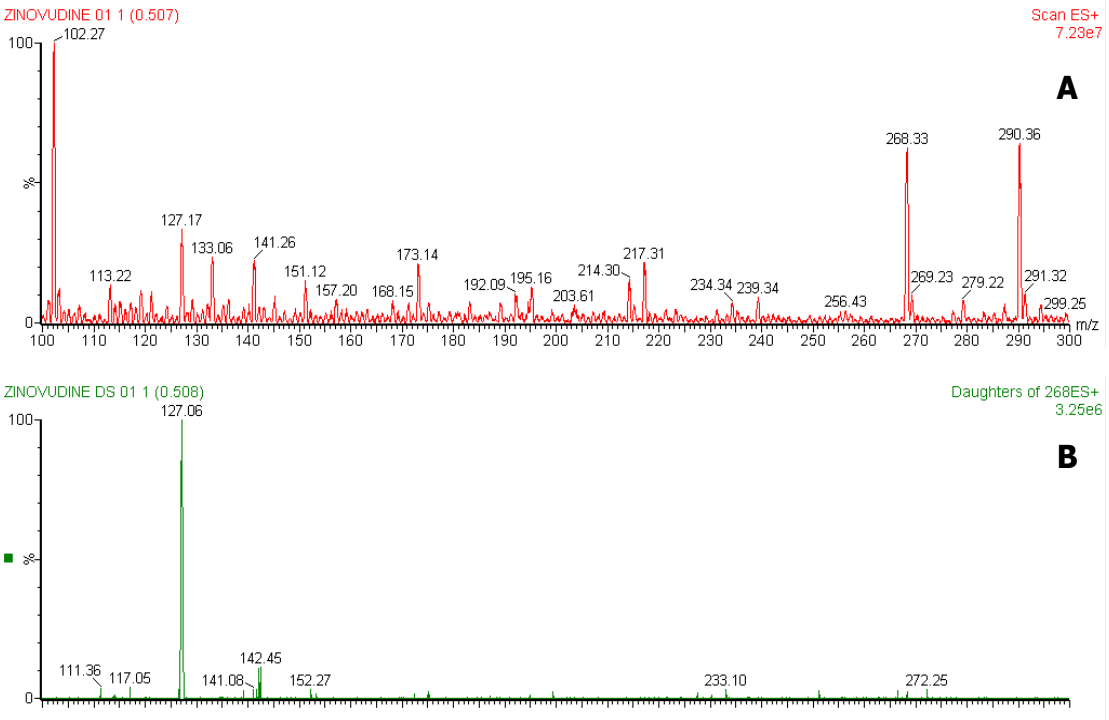

Figura 6-5. Espectro de masas para la zidovudina (A) y fragmentos obtenidos tras la ruptura de la molécula (B).

3.3.2. Linealidad y límite de cuantificación.

La linealidad se estudió en dos intervalos de concentraciones, 1-500 ng/mL y 500-10000 ng/mL, utilizando seis puntos en cada una de las rectas. Para estudiar la linealidad se analizaron cuatro replicados de cada recta. El área de los picos se relacionó con las concentraciones mediante una ecuación lineal del tipo:

$$y=a+bx$$

siendo *y* el área del pico cromatográfico, *x* la concentración de zidovudina en la recta patrón, *b* la pendiente de la recta y *a* la ordenada en el origen. Se utilizó un método de regresión lineal de mínimos cuadrados utilizando $1/y^2$ como factor de ponderación, y se calcularon los coeficientes de correlación (r) y determinación (r^2).

Los resultados obtenidos se muestran en la siguiente tabla:

Recta	a	p(a)	b	p(b)	r	r^2
1-500 ng/mL	224,300	0,000	468,879	0,000	0,996	0,993
500-10000 ng/mL	-2213,456	0,087	70,383	0,000	0,997	0,994

Tabla 6-9. Parámetros obtenidos tras el análisis de regresión lineal ponderada.

La técnica fue lineal en los dos intervalos con coeficientes de correlación y determinación próximos a 1 y p(a)<0,05, y proporcional para el intervalo 1-500 ng/mL (p(b)<0,05), pero no para el intervalo 500-10000 ng/mL (p(b)<0,05).

Se definió como límite de cuantificación la concentración más baja de las rectas de calibrado, 1 ng/mL, que se estimó con una buena exactitud y precisión (6 replicados C.V.<20%).

3.3.3. Exactitud.

Para el estudio de la exactitud se analizaron cinco replicados de tres concentraciones (baja, media y alta) de cada recta de calibrado. Las concentraciones se calcularon utilizando la ecuación de la recta estimada en el apartado anterior y se determinó el porcentaje de recuperación (($C_{exp}/C_{teórica}$) x100). Los resultados obtenidos se muestran en la Tabla 6-10. En todos los casos se

obtuvieron C.V. inferiores al 11%, excepto en la concentración de 1 ng/mL donde se acepta una mayor variabilidad por ser el límite de cuantificación (C.V.<20%).

Recta	Conc. Teórica (μg/mL)	% Recuperación Media ± D.E.	C.V.
1-500 ng/mL	1	103,00 ± 17,86	17,34
	50	104,24 ± 11,21	10,76
	500	99,78 ± 3,73	3,74
500-10000 ng/mL	500	104,10 ± 9,59	9,21
	2500	105,21 ± 10,83	10,29
	10000	100,30 ± 7,59	7,56

Tabla 6-10. Resultados obtenidos en el estudio de la exactitud.

3.3.4. Precisión.

La precisión intradía (repetibilidad) se estudió analizando cinco replicados de tres concentraciones de cada recta en el mismo día. Para la precisión interdía (reproducibilidad) se analizaron cinco replicados de tres concentraciones durante tres días. Los resultados obtenidos se recogen en las tablas 6-11 y 6-12. Los C.V. fueron en todos los casos inferiores al 14%, excepto en la concentración de 1 ng/mL donde se acepta una mayor variabilidad por ser el límite de cuantificación (C.V. <20%).

Recta	Conc. Teórica (ng/mL)	Respuesta Media ± D.E.	C.V.
1-500 ng/mL	1	707,25 ± 83,74	11,84
	50	24661,40 ± 2628,91	10,66
	500	234157,00 ± 27072,04	3,74
500-10000 ng/mL	500	34420,00 ± 3373,93	9,80
	2500	182902,80 ± 19049,61	10,42
	10000	703737,00 ± 53386,94	7,59

Tabla 6-11. Resultados obtenidos en el estudio de la precisión intradía.

Recta	Conc. Teórica (ng/mL)	Respuesta Media ± D.E.	C.V.
1-500 ng/mL	1	612,36 ± 91,91	15,01
	50	21592,13 ± 2966,19	13,74
	500	203345,20 ± 23847,30	11,73
500-10000 ng/mL	500	39816,60 ± 5434,13	13,65
	2500	201826,20 ± 17679,25	8,76
	10000	794737,67 ± 74369,10	9,36

Tabla 6-12. Resultados obtenidos en el estudio de la precisión interdía.

CONCLUSIÓN

Se han desarrollado tres técnicas analíticas para determinar amicacina y zidovudina en muestras biológicas. Entre ellas, hay que destacar la técnica de UPLC-MS/MS, que permite mejorar extraordinariamente la sensibilidad y diminuir el tiempo de análisis en la cuantificación de zidovudina.

Todas las técnicas utilizadas para la realización de este trabajo han demostrado una adecuada selectividad y linealidad en los intervalos de concentración estudiados para los fármacos analizados.

Los coeficientes de variación (C.V.) obtenidos en los estudios de la exactitud y precisión, tanto intradía como interdía, presentan valores inferiores al 15%, con excepción del límite de cuantificación para el que se admite una mayor variabilidad, aceptándose C.V. hasta del 20%.

Por lo tanto, los métodos analíticos desarrollados permiten la cuantificación precisa y exacta de amicacina y zidovudina en las muestras analizadas, siguiendo los criterios establecidos por la FDA.

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CAPÍTULO 8

Discusión

La finalidad de esta tesis doctoral es la de desarrollar y evaluar *in vitro* e *in vivo* la utilización de portadores biológicos, concretamente eritrocitos autólogos, para incrementar la selectividad en la captación de fármacos antibióticos y antirretrovirales por macrófagos.

Las células fagocíticas constituyen componentes esenciales del sistema inmunológico, siendo su principal función ingerir y destruir microorganismos. Existen, en la circulación sanguínea, diferentes tipos de células fagocíticas como leucocitos polimorfonucleares (PMN) y monocitos. Estos últimos, cuando abandonan la circulación y penetran en los tejidos se convierten en macrófagos residentes. Algunos microorganismos patogénicos, son capaces de sobrevivir y reproducirse después de haber sido ingeridos por las células fagocíticas, especialmente los macrófagos. La localización intracelular de estos microorganismos los protege de los mecanismos de defensa y de la acción de los antibióticos, que pueden encontrar dificultades para penetrar en las células fagocíticas.

Por otra parte, el sistema monocito-macrófago juega un papel fundamental en la infección por VIH ya que es una de las primeras células infectadas por el virus. A diferencia de los linfocitos CD4 activados, que mueren al ser infectados, los macrófagos no sufren alteraciones significativas en su homeóstasis, por lo que son capaces de soportar la replicación intracelular del virus actuando como reservorio y favoreciendo la diseminación de la infección por el sistema inmunitario y otros órganos, llegando al sistema nervioso central. Esto protege al virus frente a la terapéutica con fármacos antirretrovirales, de manera que el virus activo reaparece cuando se retira el tratamiento (Hu et al. 2000, Fraternale et al. 2002).

Existen una gran variedad de fármacos activos contra el VIH que evitan su replicación por distintos mecanismos, pero debido a sus propiedades físico-químicas presentan una insuficiente penetración en macrófagos y una baja biodisponibilidad, por lo que no se consiguen niveles adecuados en el lugar de infección-replicación del virus. Esto va a dar lugar a la aparición de resistencias y diversos efectos tóxicos por la acumulación del fármaco en otros órganos (von Briesen 2000).

En el amplio campo de la administración de medicamentos, los vectores o portadores ocupan un lugar destacado ya que van a permitir, utilizando estrategias muy diferentes, vehiculizar los fármacos y otras sustancias al lugar de acción o la célula diana de forma selectiva. En este área de la vectorización de fármacos se incluyen desde los sistemas más conocidos como los liposomas, las micro y nanopartículas, ~~o~~ los más recientes polímeros como los dendrímeros, hasta sistemas alternativos como los portadores biólogicos de fármacos como los basados en las lipoproteínas plasmáticas de baja densidad (LDL), los péptidos de origen natural, las células y células modificadas, las células madre y los vectores virales. Como se puede observar en la figura 1, en el campo de la terapia génica para el tratamiento del VIH se pueden utilizar desde sistemas portadores más clásicos como los liposomas o las nanopartículas hasta portadores biológicos como péptidos, sistemas celulares o vectores virales.

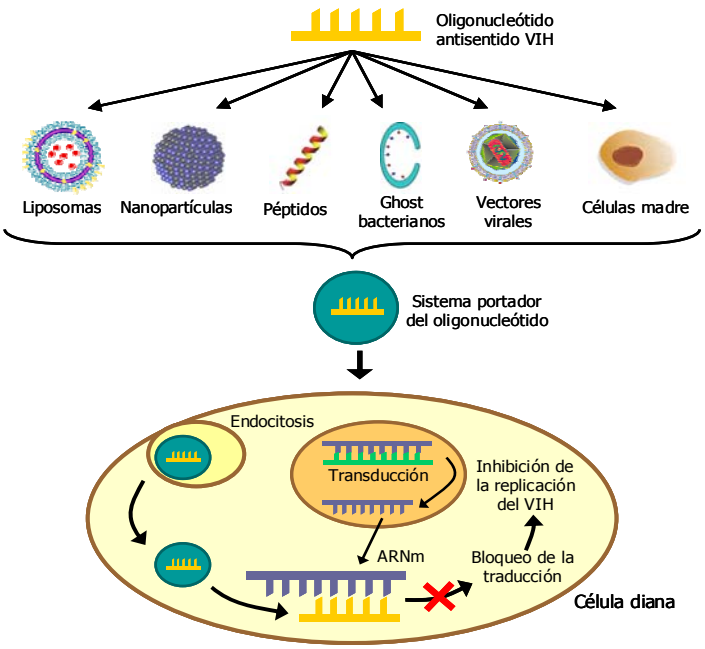

Figura 1. Sistemas portadores de oligonucleótidos antisentido para el tratamiento del SIDA (Adaptado de Lanao et al. 2007)

Diversos estudios muestran la utilidad de sistemas portadores no biológicos, como liposomas y nanopartículas, o biológicos, como los eritrocitos, para incorporar

agentes antiinfecciosos, antibióticos o antirretrovirales, al interior celular. Estos sistemas van a ser ingeridos por las células fagocíticas, acumulándose grandes cantidades de fármaco en órganos del sistema retículo endotelial (SRE), donde frecuentemente residen y se multiplican las bacterias patogénicas o el virus. De esta manera se consigue disminuir las dosis administradas, mejorando la eficacia y disminuyendo la toxicidad.

Los portadores biológicos de fármacos constituyen una interesante alternativa a otros sistemas más clásicos como los liposomas o las nanopartículas en el campo de la administración y vectorización de fármacos. Entre sus principales ventajas están su biocompatibilidad y alta especificidad, ya que permiten dirigir selectivamente el material encapsulado a diferentes órganos, tejidos y células, pudiendo vehiculizar fármacos, enzimas y material genético. Como puede observarse en la tabla 1, existen distintos tipos de portadores biológicos, entre los que deben destacarse los portadores celulares. Actualmente, este tipo de portadores presentan dianas terapéuticas muy diversas y aplicaciones potenciales en campos como la terapia del cáncer, enfermedades cardiovasculares, parkinson, AIDS, terapia génica, etc.

Portador biológico	**Diana**	**Producto encapsulado**
LDL	Macrófagos	Fármacos, péptidos
Péptidos	Dianas celulares, macrófagos	Material genético
Ghosts bacterianos	Tejidos, macrófagos, células	Fármacos, vacunas, material genético
Eritrocitos	Macrófagos, SRE	Fármacos, enzimas, péptidos
Células madre modificadas	Células tumorales, células T, macrófagos	Material genético
Leucocitos polimorfonucleares	Tejidos	Fármacos
Células apoptópicas	Células tumorales	Fármacos
Células tumorales	Células tumorales	Fármacos
Células dendríticas	Células T	Fármacos
Vectores virales	Células T	Material genético

Tabla 1. Distintos tipos de sistemas portadores biológicos (Adaptada de Lanao y Sayalero 2006).

El primer objetivo de este trabajo ha sido el de realizar una completa revisión bibliográfica sobre la penetración de agentes antiinfecciosos en células fagocíticas y el incremento en la selectividad de antibióticos y antirretrovirales por este tipo de células utilizando distintos tipos de sistemas portadores, con especial énfasis en el uso de portadores biológicos y especialmente portadores celulares como los eritrocitos de rata.

Los resultados de este estudio bibliográfico han sido publicados por la autora de esta tesis doctoral en las revistas *Journal of Controlled Release* (Briones et al. 2008) y *Journal of Drug Targeting* (Lanao et al. 2007) y se encuentran recogidos íntegramente en los capítulos 2 y 3 de esta memoria.

El segundo objetivo de este trabajo ha sido la puesta a punto y validación de distintas técnicas analíticas que permitan la cuantificación de los fármacos estudiados en diferentes tipos de muestras biológicas, como eritrocitos, macrófagos, plasma y tejidos. Se han puesto a punto varias técnicas cromatográficas, concretamente, una técnica de HPLC-fluorescencia para la cuantificación de amicacina, una técnica de HPLC-ultravioleta para la cuantificación de zidovudina en eritrocitos, y una técnica de UPLC-MS/MS para la cuantificación de zidovudina en plasma y tejidos.

Entre ellas, las nuevas técnicas de UPLC (cromatografía líquida de ultrapresión o ultrarápida) permiten trabajar con presiones muy altas de hasta 1000 bar y utilizan como fase estacionaria partículas porosas inferiores a 2 µm que mantienen la capacidad de retención y carga debido a su alta superficie. De este modo, se mejora la eficiencia en la separación y se reduce considerablemente la anchura de los picos y la duración del análisis.

Los sistemas de UPLC se combinan frecuentemente con detectores de espectrometría de masas en los que los analitos son identificados y cuantificados según su relación masa/carga. Esto permite aumentar la especificidad, gracias a la detección de los productos según su masa molecular o su patrón de ruptura y

tienen un amplio rango de aplicación con buena practicabilidad, si bien presentan el inconveniente de una mayor variabilidad.

La combinación de estas dos técnicas mejora enormemente la especificidad y reduce el tiempo de análisis y el consumo de muestra. El análisis de zidovudina mediante esta técnica ha permitido mejorar el límite de cuantificación y reducir el tiempo de retención de 3,6 minutos a 0,95 minutos.

Los resultados correspondientes a la puesta a punto y validación de las tres técnicas analíticas utilizadas se encuentran recogidas en el capítulo 7 de esta memoria. En la tabla 2 se muestra un resumen de las condiciones utilizadas en las mismas.

Condiciones cromatográficas	**Técnica analítica**		
	Cuantificación de amicacina mediante HPLC	**Cuantificación de AZT mediante HPLC**	**Cuantificación de AZT mediante UPLC**
Tratamiento de muestras	Derivatización con o-phtaldialdehído	Precipitación proteica	Precipitación proteica
Columna	Purospher RP-18 5 cmx4 mm d.i., 3 λm	Purospher RP-18 5 cmx4 mm d.i., 3 λm	UPLC BEH C18 5 cmx2,1 mm d.i., 1,7 λm
Fase móvil	Metanol/agua (62/38%) + 2,2 g/L $EDTAK_3$	KH_2PO_4 25 mM pH 7/acetonitrilo (91/9%)	Ácido fórmico 0,1%/acetonitrilo (90/10%)
Flujo	1,5 mL/min.	1 mL/min.	0,5 mL/min.
Detección	Fluorescencia (λ_{ex}= 343 nm, λ_{em}= 460 nm)	Ultravioleta (λ= 265 nm)	MS/MS ESI+ MRM 268,2→127
Tiempo de retención	12,5 min.	3,6 min.	0,95 min.

Tabla 2. Condiciones cromatográficas para las distintas técnicas analíticas utilizadas en este trabajo.

El tercer objetivo del presente trabajo ha consistido en la puesta a punto de técnicas de encapsulación de agentes antiinfecciosos en eritrocitos de rata mediante un método de diálisis hipotónica.

Los métodos osmóticos constituyen los métodos más habituales para encapsular fármacos y otras sustancias en eritrocitos, y son los más clásicos ya que la mayoría fueron desarrollados en la década de los 70. Aunque metodológicamente existen algunas diferencias entre unos y otros métodos, el fundamento de la encapsulación es similar. Estos métodos se basan en el hinchamiento de las células acompañado de un incremento en la permeabilidad de la membrana de los eritrocitos, cuando éstos son expuestos a una solución tamponada hipotónica. De los diferentes métodos desarrollados, el más habitual es la diálisis hipotónica, que admite a su vez diferentes variantes.

Como puede observarse en la figura 2, la encapsulación de fármacos en eritrocitos mediante diálisis hipotónica se realiza en varias fases. La fase fundamental es la de diálisis en la que los eritrocitos se someten a un tampón hipotónico de baja osmolalidad. En esta fase la suspensión de eritrocitos conteniendo el fármaco se coloca en una bolsa de diálisis utilizando una membrana celulósica, previamente activada, con bajo corte de peso molecular, que se introduce en el tampón hipotónico. Posteriormente se produce una fase de estabilización y un resellado posterior.

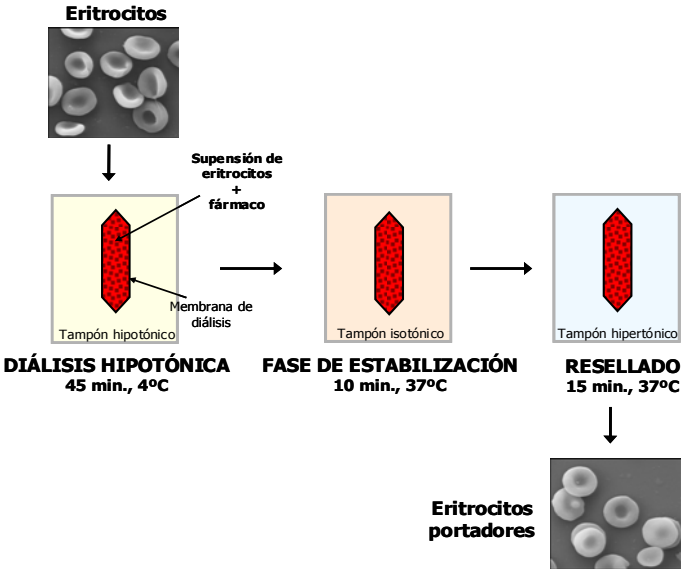

Figura 2. Procedimiento de encapsulación de fármacos en eritrocitos mediante diálisis hipotónica (Adaptado de Lanao y Sayalero 2006).

Las condiciones de encapsulación pueden ser muy variables y en la práctica hay que optimizarlas para cada sustancia a encapsular. Dependiendo de la especie animal considerada los intervalos de osmolalidad durante la diálisis hipotónica pueden ser muy variables (26-220 mOsm/Kg) con tiempos de diálisis y relaciones de volúmenes muy diferentes (Gutiérrez et al. 2004 a,b).

En los procedimientos de diálisis hipotónica resulta crítico conocer *a priori* la curva de hemólisis de los eritrocitos de la especie animal considerada, frente a la osmolalidad del medio. Osmolalidades inferiores permiten habitualmente una mayor encapsulación de sustancia, pero también inciden en la morfología del eritrocito y en una mayor fragilidad osmótica con un aumento en el porcentaje de hemólisis, así como cambios en los parámetros hematológicos de los eritrocitos.

La figura 3 muestra la relación de carácter sigmoide establecida entre el porcentaje de hemólisis y la osmolalidad del medio en eritrocitos de rata perteneciente a un grupo control.

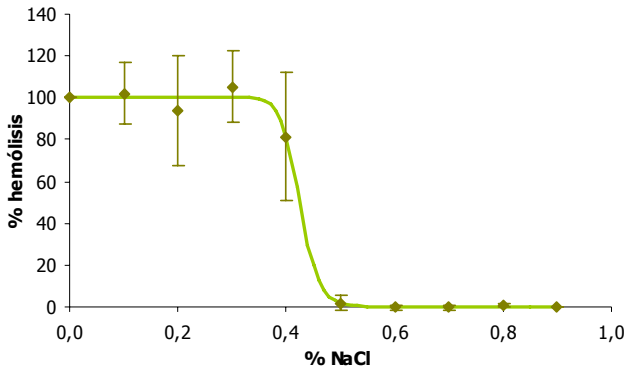

Figura 3. Relación entre la osmolalidad del medio y el porcentaje de hemólisis.

En la misma se observa cómo osmolalidades inferiores a los 300 mOsm/Kg producen un incremento significativo en la hemólisis de los eritrocitos por lo que, en la práctica, hay que buscar para cada tipo de sistema experimental una osmolalidad óptima que suponga un compromiso entre la cantidad encapsulada de fármaco y el porcentaje de hemólisis de los eritrocitos. Estudios previos realizados en nuestro

laboratorio demuestran cómo utilizando tampones hipoosmóticos de 90-100 mOsm/Kg se consigue una adecuada encapsulación de fármacos polares como los antibióticos aminoglucósidos con un aumento moderado de la fragilidad osmótica y un buen comportamiento hematológico de los eritrocitos conteniendo el fármaco (Gutiérrez et al. 2005).

Como se ha comentado anteriormente (figura 2), algunos autores proponen una fase de estabilización en condiciones isotónicas posterior a la diálisis hipotónica y previa al resellado (Grimaldi et al. 1997, Sanz et al. 1999, Gutiérrez et al. 2004b). Estudios previos realizados en nuestro laboratorio han demostrado que cuando se encapsulan sustancias de bajo peso molecular se puede prescindir de la fase de estabilización, realizando el resellado con un tampón hipertónico; de este modo disminuye la manipulación del eritrocito y la pérdida de fármaco, obteniéndose un buen rendimiento de encapsulación así como adecuadas propiedades morfológicas y hematológicas (Gutiérrez et al. 2005).

La figura 4 muestra el proceso de encapsulación de amicacina en eritrocitos de rata utilizado en este estudio. Como factores críticos en la encapsulación deben destacarse la inclusión del fármaco a altas concentraciones en el interior de la bolsa de diálisis para conseguir un gradiente favorable, la utilización de un hematocrito del 70%, la utilización de un tampón hipotónico de 90 mOsm/Kg, la relación de volúmenes de la suspensión de eritrocitos y del tampón hipotónico (1:50 mL), la ausencia de una fase de estabilización y la realización del resellado utilizando un tampón hipertónico de 500 mOsm/Kg. La composición de los tampones de diálisis y resellado constituye asimismo un factor crítico que incide tanto en el rendimiento de la encapsulación como en las propiedades de los eritrocitos portadores obtenidos. Además de sales, que influyen en la tonicidad del medio, se incluyeron en los tampones sustancias tales como glucosa, glutatión y ATP que ayudan a preservar la viabilidad de las células.

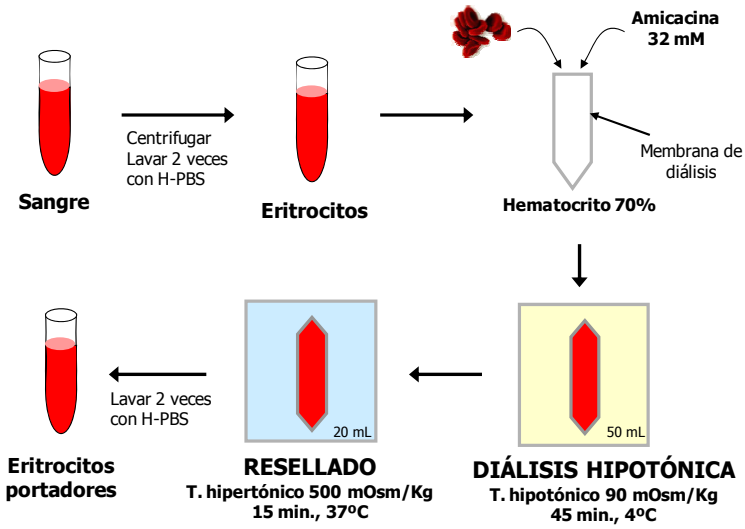

Figura 4. Proceso de encapsulación de amicacina en eritrocitos de rata.

Mediante el procedimiento descrito la cantidad encapsulada de amicacina fue 0,73 ± 0,27 mg/mL. Los eritrocitos portadores de amicacina presentaron propiedades similares a una población de eritrocitos control tanto respecto a sus parámetros hematológicos como a su fragilidad osmótica y morfología. Los estudios de liberación *in vitro* revelaron una primera fase de liberación rápida de una fracción equivalente al 30% de la cantidad encapsulada en los primeros minutos del ensayo, quedando el 70% restante retenido en el interior de las células por periodos prolongados de tiempo (Gutiérrez et al. 2005).

Otro de los objetivos planteados en esta memoria ha sido el de evaluar la captación *in vitro* por macrófagos, de fármacos antiinfecciosos utilizando eritrocitos portadores. Para ello se incubaron macrófagos peritoneales obtenidos en ratas inducidas con tioglicolato hasta obtener una monocapa de macrófagos. El procedimiento de obtención e incubación *in vitro* de los macrófagos peritoneales se describe en el capítulo 4 de esta memoria. Un esquema del procedimiento de incubación se encuentra recogido en la figura 5.

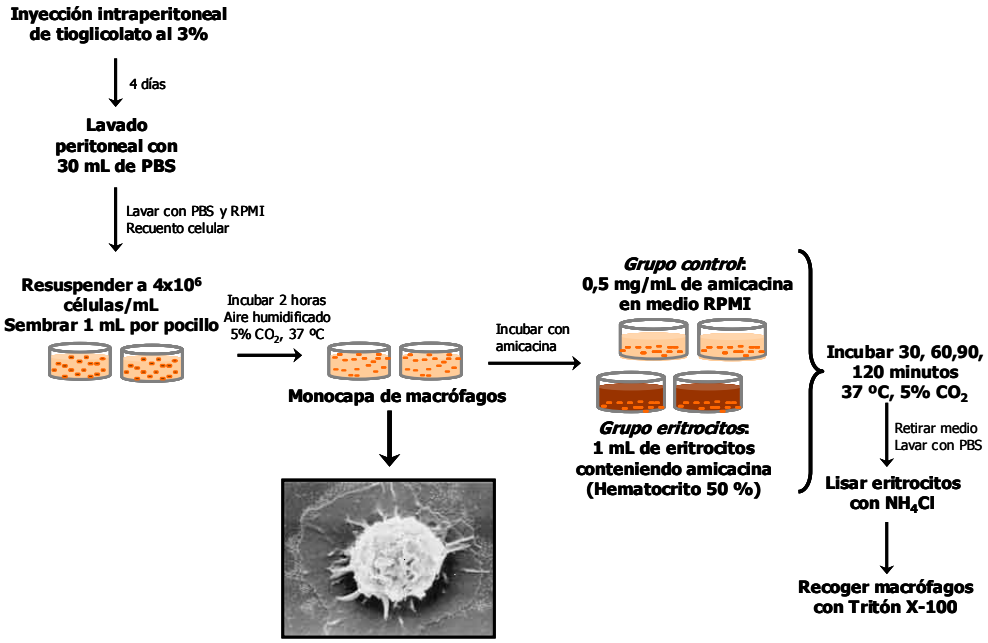

Figura 5. Procedimiento de aislamiento e incubación de macrófagos peritoneales de rata.

La figura 6 muestra las concentraciones de amicacina obtenidas en macrófagos *in vitro* después de incubarlos con el antibiótico en disolución o encapsulado en eritrocitos portadores. En la misma se observan diferencias estadísticamente significativas ($p<0,05$) a los 30 y 60 minutos de la administración, reflejando una mayor captación del antibiótico por células fagocíticas cuando se administra incorporado en los eritrocitos.

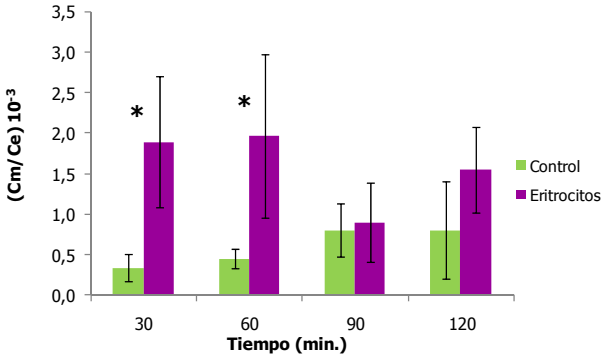

Figura 6. Concentraciones de amicacina (± D.E.), normalizadas por la dosis, en macrófagos *in vitro* tras su administración en solución o encapsulada en eritrocitos (*$p<0,05$).

Al tratarse los aminoglucósidos de antibióticos de naturaleza polar, presentan limitaciones para acceder al interior de la célula fagocítica por mecanismos pasivos de difusión simple como lo hacen los beta-lactámicos, los macrólidos o las fluoroquinolonas y utilizan principalmente mecanismos de endocitosis fluida para penetrar en el interior de la célula fagocítica. En cualquier caso su capacidad de penetración es muy baja y esto puede mejorarse a través del uso de portadores o vectores específicos. La vectorización puede ser un proceso pasivo al comportarse los vectores como sustancias extrañas al organismo que son reconocidas como tales por los macrófagos y fagocitadas, o activo, basada en modificaciones en la superficie de los portadores que facilitan la afinidad e interacción por la célula diana.

Los portadores biológicos, principalmente celulares, como los eritrocitos o los *ghosts* bacterianos presentan una alta selectividad potencial por el sistema retículo endotelial y especialmente por las células fagocíticas, al ser este el último eslabón de su ciclo biológico lo que unido a su alta biocompatibilidad los hace candidatos idóneos para la vectorización de sustancias terapéuticamente áctivas, como los fármacos antiinfecciosos, que deben actuar de forma selectiva en el interior de la célula fagocítica.

Estudios previos realizados con antibióticos aminoglucósidos demuestran que la mayor parte del fármaco encapsulado permanece retenido en los eritrocitos durante períodos prolongados de tiempo, permitiendo la liberación sostenida del antibiótico desde los eritrocitos, así como la distribución selectiva al sistema retículo endotelial (Eichler et al. 1986, Gutiérrez et al. 2005, Gutiérrez et al. 2008a). Los resultados *in vitro* en relación con la acumulación de amicacina utilizando monocapas celulares de macrófagos peritoneales (figura 6) demuestran que los eritrocitos son fagocitados por los macrófagos permitiendo una elevada acumulación del antibiótico por este tipo de células fagocíticas. Sin embargo, la acumulación intracelular no siempre se correlaciona con la actividad intracelular, ya que la actividad antibacteriana puede verse influida por factores físico-químicos, el estado metabólico del patógeno y el efecto del antibiótico en la capacidad fagocítica de la

célula (Barcia-Macay et al. 2006, Lutwyche et al. 1998, Schiffelers et al. 2001, Lanao et al. 2007, Briones et al. 2008).

Una vez demostrada la mayor captación de amicacina por macrófagos peritoneales *in vitro* cuando se utilizan eritrocitos portadores, se estudió la penetración *in vivo* de amicacina utilizando eritrocitos portadores, así como las modificaciones en la farmacocinética y en la biodistribución del antibiótico en ratas.

Para realizar este estudio se utilizaron 56 ratas Wistar, inducidas con tioglicolato al 3% por vía intraperitoneal (i.p.), repartidas en dos grupos de estudio. Un grupo recibió 1 mL de eritrocitos portadores con una dosis de 2,82 $\pm$ 1,06 mg/Kg por vía i.p, y el grupo control (n=28) recibió la amicacina a la dosis de 7,5 mg/Kg por vía i.p. A tiempos previamente programados se sacrificaron los animales, tomando muestras de fluido peritoneal, plasma, tejidos (hígado, bazo, pulmón y riñón) y macrófagos obtenidos mediante lavado peritoneal.

La figura 7 muestra comparativamente los niveles de amicacina normalizados por la dosis obtenidos tras la administración del fármaco en disolución acuosa e incorporado en eritrocitos portadores.

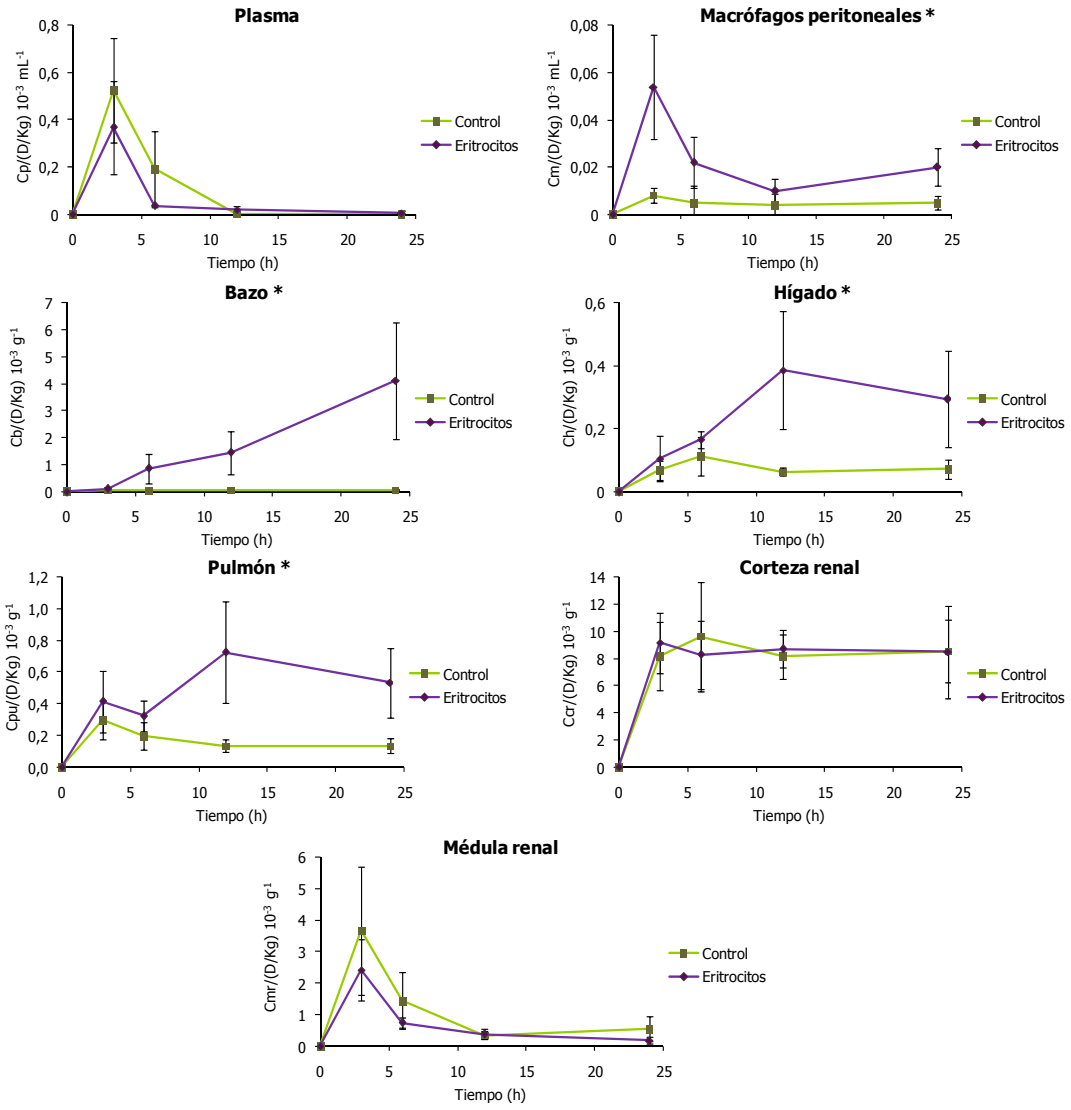

Figura 7. Concentraciones de amicacina, normalizadas por la dosis (mg/Kg), obtenidas en plasma, macrófagos y tejidos *in vivo*, tras su administración en eritrocitos portadores y en solución (* $p < 0,001$).

El análisis estadístico no paramétrico de las concentraciones normalizadas mostró diferencias estadísticamente significativas en macrófagos peritoneales, bazo, hígado y pulmón ($p<0,001$).

La tabla 3 muestra los valores de área bajo la curva (ABC_{0-24}), tiempo medio de residencia (TMR_{0-24}), y el coeficiente de reparto (R) de amicacina *in vivo* en el grupo control y en el grupo de eritrocitos portadores. Como se observa en la tabla, cuando la amicacina se administra incorporada en eritrocitos portadores se produce un incremento en el área bajo la curva y/o en el coeficiente de reparto en macrófagos peritoneales, hígado, bazo y pulmón en comparación con el grupo control.

Tejido	Control			Eritrocitos		
	$(ABC)_{0-24}$ **((μg/g)/(mg/Kg)) h**	**$(TMR)_{0-24}$** **(h)**	**R**	**$(ABC)_{0-24}$** **((μg/g)/(mg/Kg)) h**	**$(TMR)_{0-24}$** **(h)**	**R**
Plasma	2,11*	4,26		1,29*	5,39	
Macrófagos	0,11*	12,13	0,05	0,46*	10,97	0,36
Hígado	1,71	12,26	0,81	6,29	14,26	4,88
Bazo	1,43	13,30	0,68	41,96	18,47	32,52
Pulmón	3,77	11,11	1,79	12,38	13,56	9,59
Corteza renal	192,77	12,71	91,32	194,48	12,73	150,76
Médula renal	22,74	8,24	10,78	14,50	7,65	11,24

* ((μg/mL)/(mg/Kg)) h

Tabla 3. Parámetros farmacocinéticos modelo-independiente de amicacina obtenidos en los grupos control y eritrocitos portadores.

Las figuras 8 y 9 muestran la captación relativa de amicacina en macrófagos peritoneales y tejidos *in vivo* utilizando la relación entre el coeficiente de reparto y el área bajo la curva obtenidos en el grupo eritrocitos y el grupo control. La captación relativa de amicacina obtenida fue: bazo > macrófagos peritoneales > hígado > pulmón > corteza renal > médula renal.

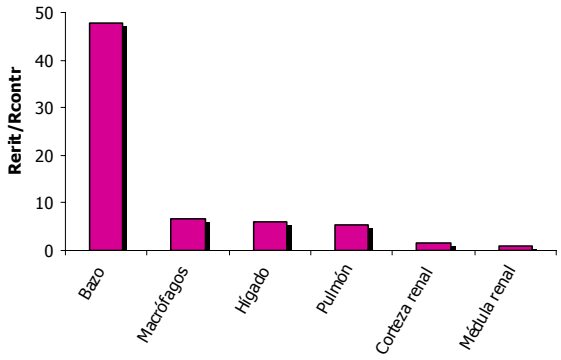

Figura 8. Captación relativa de amicacina en macrófagos y tejidos *in vivo* utilizando la relación entre los coeficientes de reparto en tejidos de los grupos eritrocitos y control.

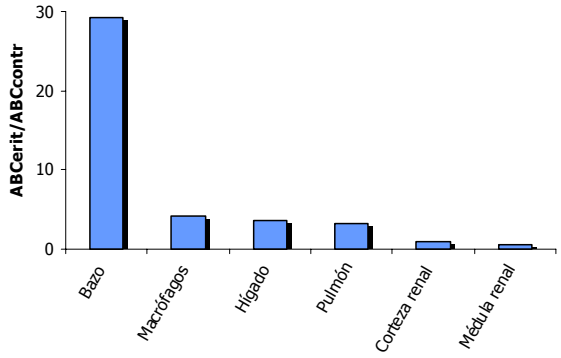

Figura 9. Captación relativa de amicacina en macrófagos y tejidos *in vivo* utilizando la relación entre el área bajo la curva en tejidos de los grupos eritrocitos y control.

La cinética plasmática de amicacina después de la administración i.p de eritrocitos conteniendo este fármaco demostró un efecto de liberación sostenida, con un incremento en la semivida plasmática del antibiótico desde 1,27 horas en el grupo control hasta 6,36 horas en el grupo de eritrocitos portadores. Como se observa en las figuras 8 y 9, la farmacocinética tisular de amicacina *in vivo* demostró una mayor acumulación del antibiótico en el bazo, macrófagos peritoneales e hígado y cambios moderados de la farmacocinética en el pulmón, corteza y médula renal, como se aprecia a su vez en la tabla 3. Por otra parte, la mayor acumulación del antibiótico en macrófagos peritoneales observada *in vivo*

cuando se administraron los eritrocitos portadores está en concordancia con los resultados obtenidos *in vitro* utilizando monocapas celulares de macrófagos.

Además, se ha observado un importante incremento en los niveles de amicacina en bazo e hígado como se observa en la figura 7. Un estudio previo sobre farmacocinética de amicacina en ratas utilizando eritrocitos portadores administrados por vía i.v., demostró cambios en la farmacocinética del antibiótico incorporado en este tipo de sistema portador. Estos cambios implicaron un incremento en la semivida y en el tiempo medio de tránsito así como una mayor acumulación del antibiótico en hígado y bazo, que son dos órganos fundamentales del sistema inmunológico (Gutiérrez et al. 2008b).

El hígado contiene células fagocíticas del tipo de linfocitos y monocitos, que capturan bacterias desde la sangre cuando ésta pasa a través del órgano. El bazo también contiene linfocitos y monocitos. Los monocitos son leucocitos agranulares que se transforman en macrófagos constituyendo el sistema monocito-macrófago. Tanto los monocitos como los macrófagos constituyen células fagocíticas. Los monocitos no tienen una función específica, sino que constituyen una reserva móvil de células. Cuando abandonan los capilares hacia los tejidos aumentan su tamaño y capacidad fagocítica, transformándose en macrófagos, que pueden desplazarse o permanecer en los tejidos.

El aumento en los niveles de amicacina observado en el hígado y bazo de los animales tratados con eritrocitos portadores se correlaciona con la mayor acumulación de amicacina en macrófagos observada tanto *in vitro* como *in vivo*.

La mayor selectividad del antibiótico por el sistema retículo endotelial y la mayor acumulación del antibiótico en macrófagos cuando se administra incorporado en eritrocitos demuestran la utilidad potencial de este tipo de sistema portador para el tratamiento de infecciones intracelulares.

La metodología y resultados de este estudio de captación *in vitro* e *in vivo* de amicacina en macrófagos han sido enviados para su publicación a la revista

European Journal of Pharmaceutical Sciences, y aparecen recogidos en el capítulo 4 de este trabajo.

La zidovudina (AZT) fue el otro fármaco antiinfeccioso utilizado en esta tesis doctoral como modelo para su encapsulación en eritrocitos y el estudio de su penetración en macrófagos y otros tejidos. El AZT es un derivado de la pirimidina que se utiliza como fármaco antirretroviral inhibiendo la replicación de los retrovirus.

El AZT fue seleccionado como fármaco antiinfeccioso alternativo a la amicacina considerando que se trata de un fármaco altamente apolar, a diferencia de la amicacina que constituye un antibiótico polar de alta solubilidad en agua. En ambos fármacos la utilización de eritrocitos portadores puede presentar un potencial interés terapéutico considerando la importancia de la localización de ambos fármacos en el interior de las células fagocíticas.

La figura 10 muestra el proceso de encapsulación de zidovudina en eritrocitos de rata utilizado en este estudio. El método, basado también en la diálisis hipotónica, constituye una adaptación del utilizado para la amicacina. Consta de dos fases fundamentales, una de diálisis basada en la utilización de un tampón hipotónico y otra de resellado basada en la utilización de un tampón hipertónico. En este método se prescindió también de una fase de estabilización de los eritrocitos posterior a la de diálisis hipotónica.

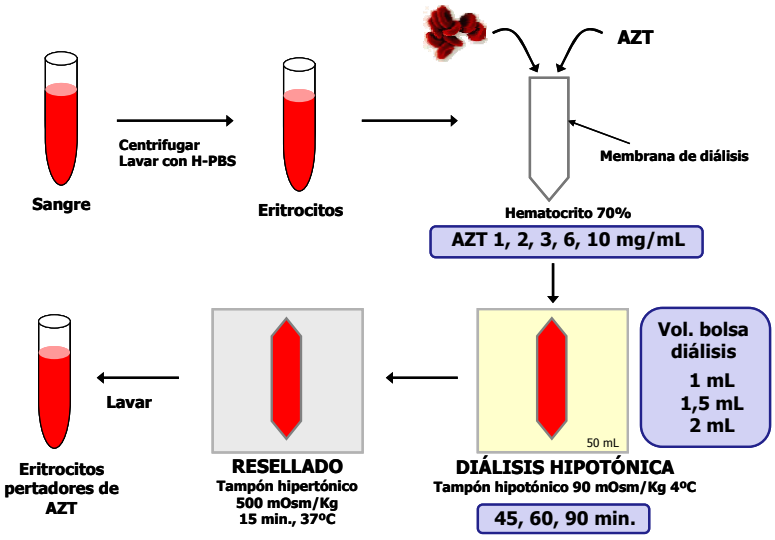

Figura 10. Procedimiento de encapsulación de AZT en eritrocitos de rata, y diferentes condiciones estudiadas para su puesta a punto.

La figura 11 recoge las micrografías obtenidas mediante microscopía electrónica de barrido (SEM), que muestran los cambios morfológicos en el eritrocito como consecuencia del proceso de encapsulación de zidovudina mediante diálisis hipotónica. Estudios previos (Kruse 1991, Gutiérrez et al. 2004a), realizados mediante microscopía electrónica de barrido (SEM) demuestran que los eritrocitos obtenidos mediante diálisis hipotónica, cambian inicialmente su morfología de discocitos a equinocitos, para posteriormente transformarse en tipos celulares morfológicamente heterogéneos.

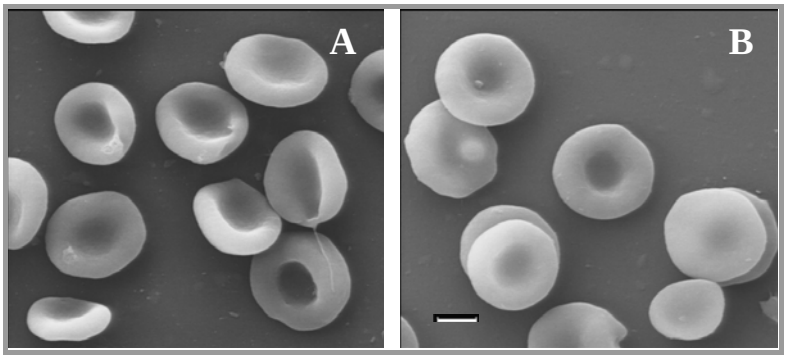

Figura 11. SEM micrografías de eritrocitos control (A) y eritrocitos portadores de AZT (B) (500 MAG) (— 2 μm).

Al ser la primera vez que se encapsulaban fármacos antirretrovirales en nuestro laboratorio, se procedió a identificar los factores críticos que podían afectar a la encapsulación de AZT en los eritrocitos y, de esta forma, optimizar el procedimiento de encapsulación. Las condiciones ensayadas fueron:

1) Concentraciones iniciales de AZT en el intervalo de 1 a 10 mg/mL para un tiempo de diálisis de 45 minutos y una relación de volúmenes entre la bolsa de diálisis y el medio tamponado de 1:50 mL.

2) Tiempos de diálisis en el intervalo de 45 a 90 minutos para una concentración inicial de AZT de 10 mg/mL y una relación de volúmenes entre la bolsa de diálisis y el medio tamponado de 1:50 mL.

3) Volúmenes en la bolsa de diálisis entre 1 y 2 mL de suspensión de eritrocitos, frente a un volumen fijo de tampón hipotónico de 50 mL, para una concentración inicial de 10 mg/mL y un tiempo de diálisis de 45 min.

Los resultados obtenidos muestran que elevadas concentraciones de AZT, tiempos de diálisis prolongados y un mayor volumen de suspensión celular en la bolsa de diálisis mejora la encapsulación de AZT en los eritrocitos. El estudio estadístico de los datos mediante un análisis de la varianza (ANOVA) ha demostrado diferencias estadísticamente significativas en la cantidad de AZT encapsulado para los diferentes factores evaluados.

La tabla 4 muestra asimismo los resultados del análisis de regresión multilineal. La cantidad de AZT encapsulado fue considerada la variable dependiente y la concentración inicial de AZT, el tiempo de diálisis y el volumen de la bolsa de diálisis fueron consideradas como variables independientes.

Parámetro	Valor	Límites de confianza (95%)		Error estándar	p	r
Constante	-909,510	-1241,060	-577,960	166,357	< 0,001	
Conc. inicial AZT (mg/mL)	80,882	62,411	99,353	9,268	< 0,001	0,855
Tiempo de diálisis (min)	10,983	6,439	15,528	2,280	< 0,001	0,454
Volumen bolsa de diálisis (mL)	569,578	371,289	767,868	99,493	< 0,001	0,545

Tabla 4. Resultados de la regresión lineal múltiple entre la cantidad de amicacina encapsulada en eritrocitos y la concentración inicial de AZT, el tiempo de diálisis y el volumen de la bolsa de diálisis.

La figura 12 (A, B y C) muestra el resultado de la regresión lineal múltiple establecida entre la cantidad de AZT encapsulada en los eritrocitos con la concentración inicial de AZT, con el tiempo de diálisis, y con el volumen de la bolsa de diálisis respectivamente, generadas utilizando la ecuación de regresión múltiple incluida en la tabla 4.

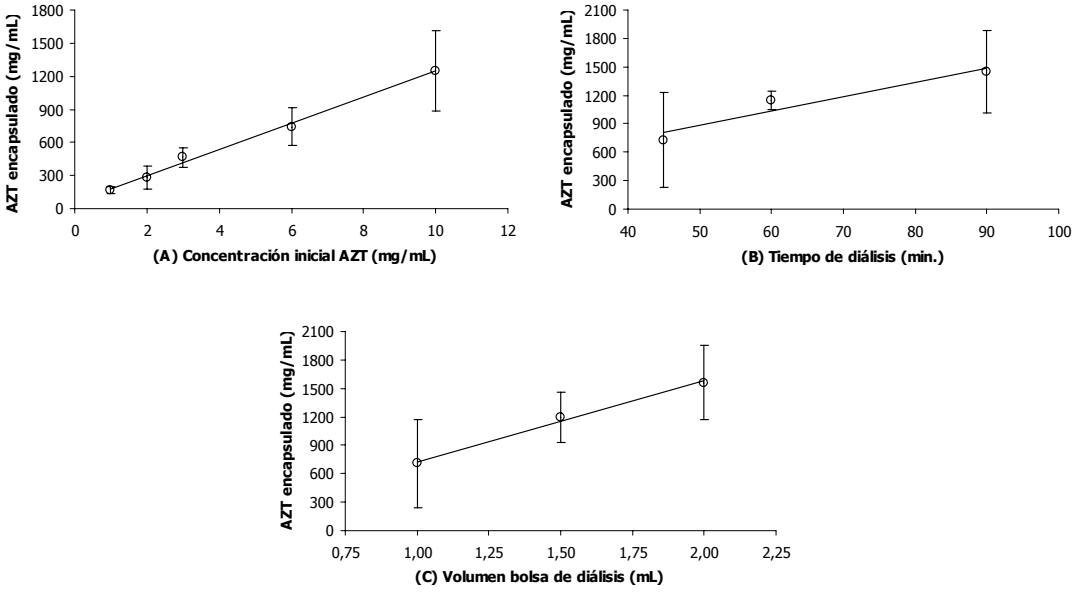

Figura 12. Regresión lineal entre la cantidad de AZT encapsulado y los diferentes factores ensayados.

Considerando el carácter lipófilo de la zidovudina, la captación del fármaco por eritrocitos puede ser, parcialmente, un proceso de convección a través de los poros inducidos por un tampón hipotónico, y en parte un proceso de difusión pasiva a través de membrana. De acuerdo con los resultados recogidos en la tabla 4, un incremento en la concentración de fármaco en la bolsa de diálisis facilitará la encapsulación en los eritrocitos a través de un proceso pasivo (Lotero et al. 2003; Murray et al. 2006). Otro factor fundamental en el proceso de encapsulación del fármaco es la duración de la diálisis. Este tiempo es variable dependiendo de las diferentes sustancias a encapsular y varía en intervalos que van desde los 20 a los 180 minutos, aunque tiempos de diálisis entre 45 y 75 minutos suelen ser los más habituales (Gutiérrez et al. 2004a,b). En este estudio se ha demostrado una mayor captación de AZT a medida que se utilizan tiempos de diálisis más prolongados, si bien tiempos muy largos de diálisis podrían afectar a la viabilidad de las células, así como a sus parámetros hematológicos. La influencia de factores como la alta concentración inicial de fármaco o tiempos prolongados de diálisis hipotónica en la eficiencia de la encapsulación, habían sido previamente descritos con amicacina por nuestro grupo de investigación (Gutiérrez et al. 2008a). Sin embargo, la influencia de factores como la relación de volúmenes entre la bolsa de diálisis y el medio tamponado, no han sido previamente descritos. Como muestra la tabla 4, un volumen en la bolsa de diálisis comprendido entre 1 y 2 mL, incubado frente al mismo volumen de tampón incrementa la cantidad de fármaco encapsulado. Este fenómeno puede explicarse como una alteración del gradiente del fármaco en la bolsa de diálisis. El elevado coeficiente obtenido en el análisis de regresión múltiple indica una alta sensibilidad de la cantidad encapsulada para este factor. Serían necesarios estudios posteriores trabajando con altas relaciones de volúmenes entre la bolsa de diálisis y el tampón hipotónico para confirmar estos resultados con zidovudina y otros fármacos.

El análisis inferencial de los parámetros hematológicos utilizando estadística no paramétrica, mostró diferencias estadísticamente significativas en algunos parámetros hematológicos para las diferentes condiciones de encapsulación

estudiadas y especialmente cuando se utilizaron tiempos de diálisis prolongados (≥ 60 minutos) en parámetros como HCT, MCV, MCH y MCHC ($p<0,05$).

Complementariamente al análisis inferencial se realizó análisis multivariante mediante análisis de clusters. El análisis de clusters, es una técnica estadística multivariante cuya finalidad es dividir un conjunto de variables en grupos (clusters) de forma que los perfiles de las variables en un mismo grupo sean muy similares entre sí y los de las variables de clusters diferentes sean distintos.

La utilización de análisis de clusters con las 5 variables hematológicas estudiadas, confirma los resultados del análisis inferencial. Como puede observarse en la figura 13 para una distancia Euclidea de 2 y utilizando la raíz cuadrada de las variables estudiadas, se demuestra la existencia de dos clusters discretos que reflejan un comportamiento hematológico anormal de los eritrocitos en condiciones límite, especialmente cuando se utilizan tiempos de diálisis prolongados iguales o superiores a los 60 minutos.

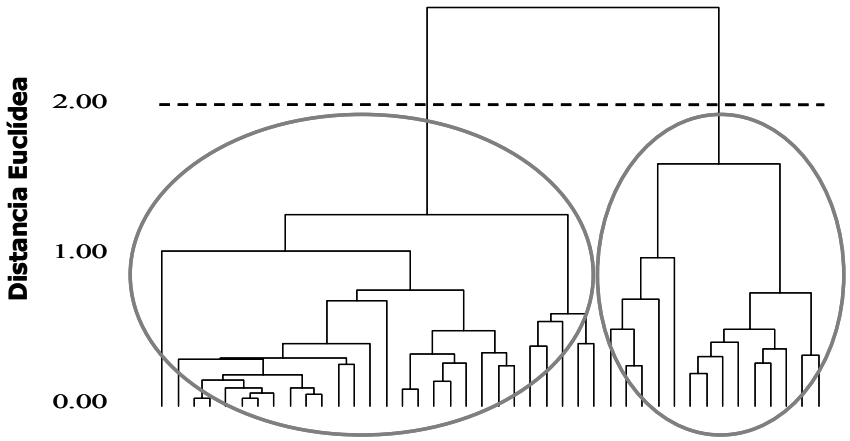

Figura 13. Análisis de clusters de los parámetros hematológicos utilizando una distancia Euclidea de 2 y la raíz cuadrada de las variables analizadas.

En cuanto a la fragilidad osmótica de los eritrocitos obtenidos en las diferentes condiciones de diálisis, y como se observa en la figura 14 no se

observaron diferencias estadísticamente significativas en las curvas de fragilidad osmótica para las diferentes condiciones de diálisis ensayadas.

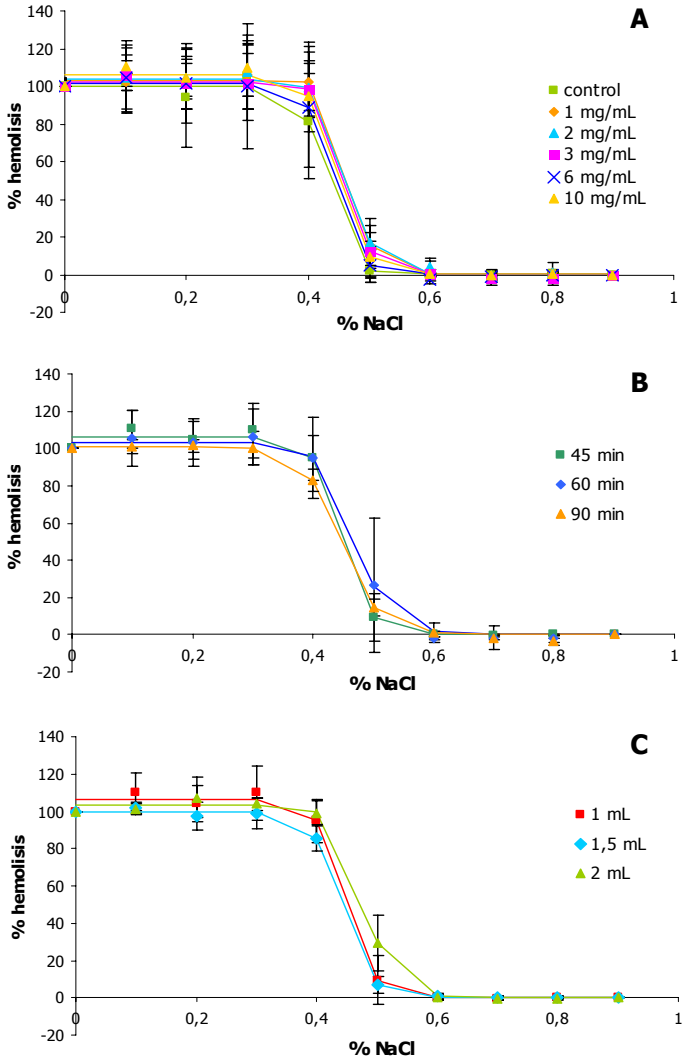

Figura 14. Curvas de fragilidad osmótica de eritrocitos control y eritrocitos portadores de AZT obtenidos utilizando diferentes condiciones.

Además, el índice de fragilidad osmótica fue sólo ligeramente superior en los eritrocitos conteniendo AZT que en los eritrocitos control como se observa en la tabla 5.

Condiciones		Índice de Fragilidad Osmótica (%)
Control		0,425
Concentración inicial de AZT	1 mg/mL	0,474
	2 mg/mL	0,464
	3 mg/mL	0,461
	6 mg/mL	0,438
	10 mg/mL	0,447
Tiempo de diálisis	45 min.	0,447
	60 min.	0,470
	90 min.	0,444
Volumen bolsa de diálisis	1 mL	0,447
	1,5 mL	0,438
	2 mL	0,478

Tabla 5. Índice de fragilidad osmótica obtenido para las distintas condiciones ensayadas.

La cinética de liberación de AZT desde eritrocitos portadores de rata muestra una rápida cesión de una fracción significativa de la cantidad encapsulada, permaneciendo alrededor del 30% del fármaco en los eritrocitos por períodos prolongados de tiempo, constituyendo el eritrocito un reservorio del fármaco desde el que se produce una liberación sostenida del mismo. Este comportamiento a nivel de liberación, contrasta con el observado previamente con amicacina donde el antibiótico permanece asimismo acumulado en el interior de la célula durante largos periodos de tiempo, pero donde el porcentaje de fármaco inicialmente cedido al medio es muy inferior (figura 15).

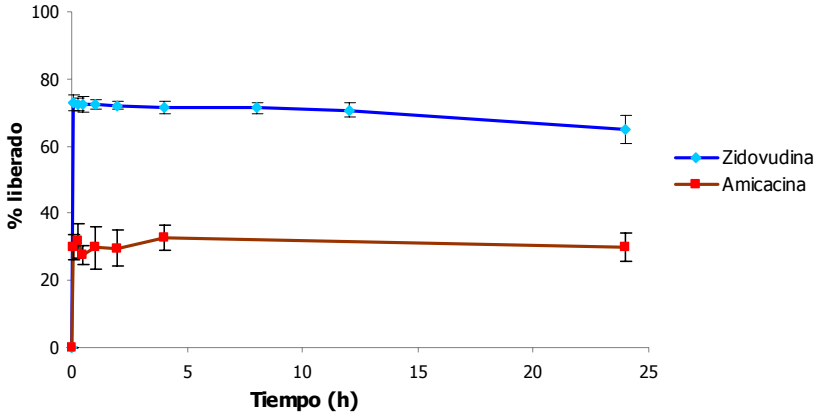

Figura 15. Liberación de zidovudina y de amicacina (Gutiérrez et al. 2005) desde eritrocitos portadores obtenidos mediante diálisis hipotónica.

Este hecho puede deberse a las diferencias de liposolubilidad entre ambos fármacos. Al tratarse el AZT de un fármaco apolar, una fracción de la dosis encapsulada puede abandonar rápidamente el eritrocito mediante mecanismos de difusión pasiva, mientras que otra fracción de la dosis permanece fijada a estructuras intracelulares y es responsable de la liberación sostenida del fármaco. La amicacina sin embargo, al tratarse de un fármaco polar, una vez encapsulada en el eritrocito tiene dificultades para abandonar la célula por mecanismos de difusión, de aquí que la pérdida inicial de fármaco sea muy inferior.

La diamida constituye una sustancia que induce la formación de puentes disulfuro en las proteínas de la superficie celular, lo que provoca cambios estructurales en la misma que pueden afectar a la permeabilidad de la membrana. El uso de diamida ha sido sugerido por algunos autores para incrementar la selectividad de los eritrocitos por el sistema retículo-endotelial y reducir la pérdida de fármaco desde los eritrocitos (Lotero et al. 2001). Por esta razón la diamida fue utilizada en este estudio para evaluar su influencia en la prevención de la rápida fuga de una fracción significativa del fármaco desde los eritrocitos portadores. Como se observa en la figura 16 el tratamiento previo de los eritrocitos con diamida no previno la rápida liberación del fármaco, no existiendo diferencias estadísticamente

significativas en las cantidades de fármaco liberadas desde los eritrocitos (p>0,05) con y sin tratamiento previo con diamida.

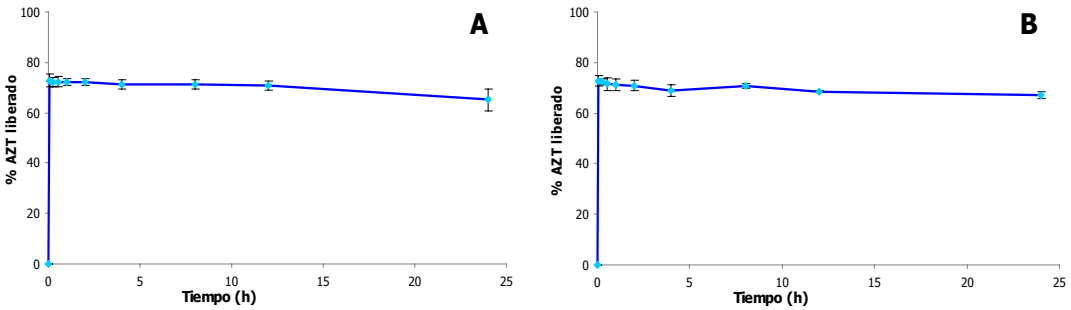

Figura 16. Liberación de AZT desde eritrocitos portadores (A) y eritrocitos portadores sometidos a un tratamiento previo con diamida (B).

Las condiciones finalmente seleccionadas como resultado de la optimización del proceso de encapsulación de zidovudina en eritrocitos y teniendo en cuenta el rendimiento de la encapsulación, las propiedades hematológicas de los eritrocitos tratados y la fragilidad osmótica fueron: 10 mg/mL como concentración inicial de AZT, 45 minutos como tiempo de diálisis, y 2 mL como volumen en la bolsa de diálisis. La concentración de AZT encapsulada con estas condiciones fue 1,56 ± 0,39 mg/mL, y el rendimiento de encapsulación 15,6%.

La metodología y resultados de este estudio han sido enviados para su publicación a la revista *Internacional Journal of Pharmaceutics*, y se recogen en el capítulo 5 de esta memoria.

Una vez completado el estudio de encapsulación y evaluación de eritrocitos portadores de zidovudina *in vitro*, la siguiente fase del trabajo consistió en estudiar la captación de AZT por macrófagos peritoneales *in vivo* así como los cambios en la cinética plasmática y en la distribución tisular cuando los eritrocitos portadores son administrados por vía intraperitoneal en ratas.

Para la realización del estudio se utilizaron 64 ratas Wistar inducidas previamente con tioglicolato al 3%, que se dividieron en dos grupos de estudio

(n=32). Un grupo recibió una dosis de zidovudina de 12,71 ± 2,65 mg/Kg incorporada en eritrocitos portadores autólogos por vía i.p y se compararon los resultados obtenidos con un grupo control que recibió zidovudina a la dosis de 8 mg/Kg en solución acuosa por vía i.p. A tiempos previamente programados se sacrificaron los animales y se obtuvieron muestras de plasma, bazo, hígado, pulmón, riñón, cerebro, monocitos procedentes de la médula ósea y macrófagos peritoneales, para la cuantificación posterior del antirretroviral mediante una técnica de UPLC-MS/MS previamente validada.

La figura 17 muestra los niveles plasmáticos de zidovudina normalizados por la dosis en plasma y en los diferentes tejidos estudiados, incluyendo macrófagos peritoneales, tras la administración de zidovudina en disolución y en eritrocitos portadores.

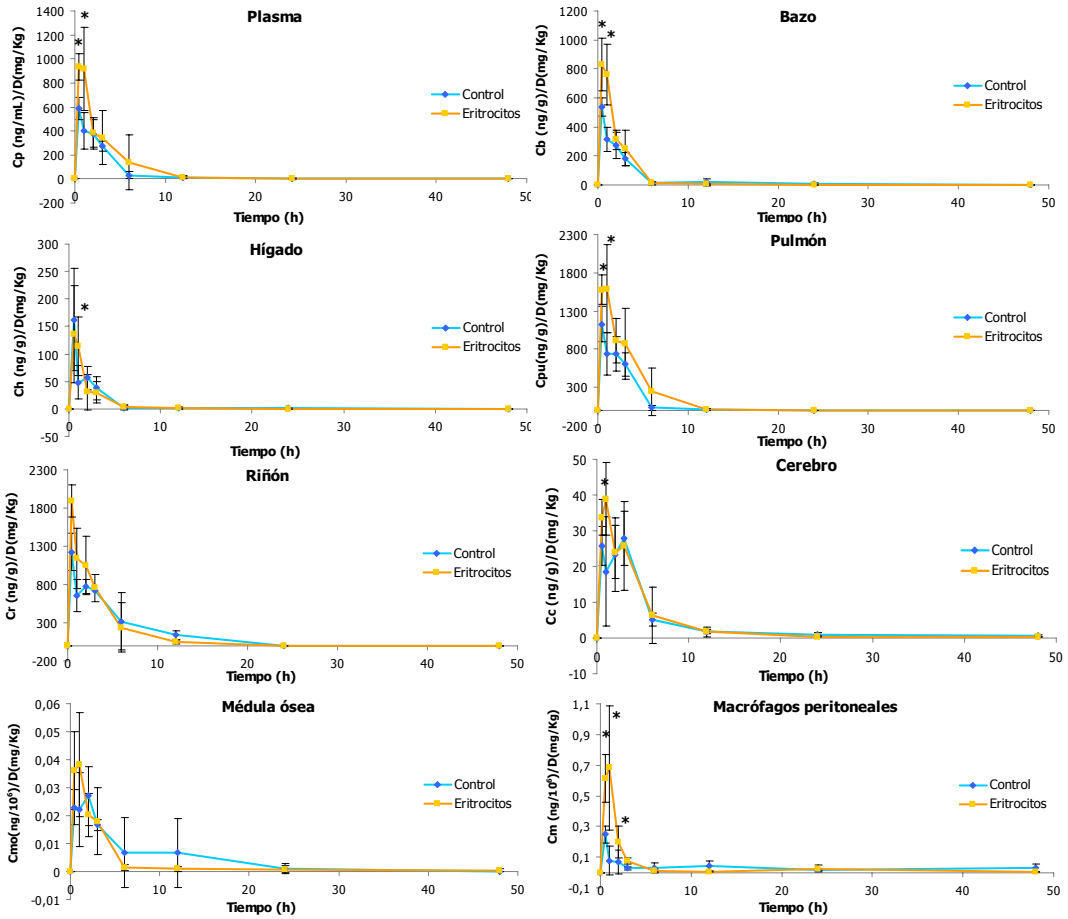

Figura 17. Niveles medios de zidovudina (± S.D.) en plasma y tejidos normalizados por la dosis tras la administración de zidovudina en solución o encapsulada en eritrocitos (* $p<0,05$).

La tabla 6 muestra, asimismo, los parámetros farmacocinéticos modelo-independiente constante de la fase terminal de primer orden (λz), semivida aparente ($t_{1/2}$), área bajo la curva ($ABC_{0-\infty}$), tiempo medio de residencia (TMR), tiempo medio de tránsito (TMT), y el coeficiente de reparto (R) de AZT, obtenidos en el grupo control y en el grupo que recibió los eritrocitos portadores.

Grupo control						
Tejido	**λz (h^{-1})**	**$t_{1/2}$ (h)**	**$(ABC)_{0-\infty}$ ((ng/g)/(mg/Kg)) h**	**TMR (h)**	**TMT (h)**	**R**
Plasma	0,108	6,44	1684,91	3,80	---	---
Bazo	0,134	5,17	1301,93	4,44	0,63	0,78
Hígado	0,133	5,22	249,89	3,24	---	0,16
Pulmón	0,193	3,59	2937,20	2,75	---	1,75
Riñón	0,258	2,69	5414,81	4,84	1,04	1,96
Cerebro	0,026	26,45	180,23	18,37	14,57	7,20E-02
Méd. ósea	0,100	6,91	0,19	8,46	4,65	5,00E-05
Macrófagos	0,047	14,65	1,17	18,32	14,52	3,11E-02
Grupo eritrocitos						
Tejido	**λz (h^{-1})**	**$t_{1/2}$ (h)**	**$(ABC)_{0-\infty}$ ((ng/g)/(mg/Kg)) h**	**TMR (h)**	**TMT (h)**	**R**
Plasma	0,142	4,86	2750,99*	3,08	---	---
Bazo	0,093	7,44	1706,55	2,24	----	0,62
Hígado	0,022	31,50	240,14	2,73	----	0,09
Pulmón	0,023	30,13	5335,09	3,17	0,09	2,02
Riñón	0,083	8,35	5459,46	3,41	0,34	1,98
Cerebro	0,032	21,79	174,24	9,54	6,46	0,05
Méd. ósea	0,023	29,74	0,17**	15,21	12,14	6,18E-05
Macrófagos	0,017	40,29	1,56**	20,17	17,09	5,67E-04

* ((ng/mL)/(mg/Kg)) h, ** ((ng/10^6cells)/(mg/Kg)) h

Tabla 6. Parámetros farmacocinéticos modelo-independientes de AZT obtenidos en los grupos control y eritrocitos portadores.

Por otra parte, las figuras 18 y 19 muestran comparativamente el coeficiente de reparto y la semivida aparente de AZT en los tejidos tras la administración del fármaco en disolución o como eritrocitos portadores.

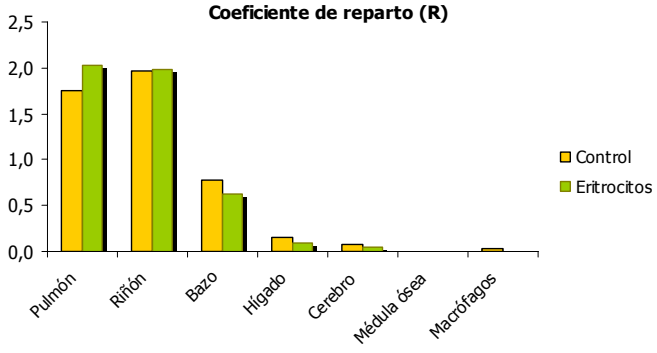

Figura 18. Coeficiente de reparto de AZT en tejidos en los grupos control y eritrocitos.

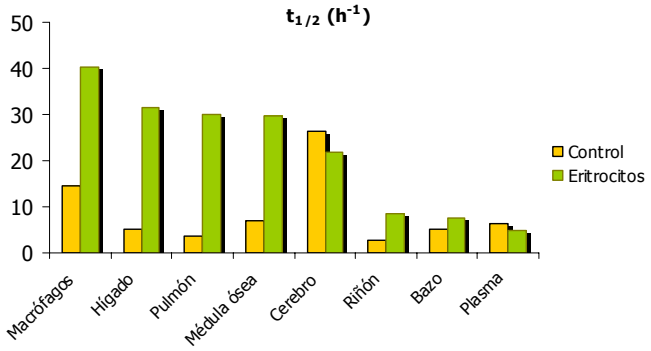

Figura 19. Semivida aparente de AZT en tejidos en los grupos control y eritrocitos.

Los resultados obtenidos en relación con la farmacocinética de AZT en el grupo control reflejan que la cinética plasmática de AZT se caracteriza por un valor de semivida de la fase terminal de alrededor de 6 horas. En este mismo grupo, se observaron diferentes concentraciones tisulares del fármaco, dependiendo del órgano o tejido específico estudiado. Como se observa en la figura 18 y en la tabla 6, el riñón, el pulmón y el bazo fueron los tejidos con un mayor coeficiente de reparto. Por el contrario, los coeficientes de reparto más bajos fueron obtenidos en macrófagos peritoneales y monocitos de la médula ósea, aunque al tratarse de células, este coeficiente de reparto no es directamente comparable ya que se ha obtenido a partir de las concentraciones de fármaco normalizadas por la dosis y por número de células. Los tejidos y células con un coeficiente de reparto más bajo

como cerebro, macrófagos peritoneales y monocitos fueron a su vez los que presentaron semividas aparentes y tiempos medios de tránsito más prolongados como demuestra la figura 19 y la tabla 6.

La infección viral de los macrófagos se considera una fase crítica en la transmisión del HIV-1. Considerando que las células fagocíticas como los macrófagos constituyen el reservorio del HIV-1, los tejidos que son ricos en macrófagos residentes como el bazo, el hígado y el pulmón constituyen dianas potenciales para la zidovudina. Los resultados obtenidos después de la administración del fármaco por vía i.p. en ratas ha demostrado una elevada acumulación de AZT en pulmón y una moderada a baja acumulación en bazo e hígado.

La administración de AZT incorporado en eritrocitos portadores modifica significativamente la farmacocinética del antirretroviral en plasma, tejidos específicos y macrófagos como demuestra la figura 17. El análisis no parámetrico de las concentraciones de AZT en plasma y tejidos en el grupo control y en el grupo que recibió los eritrocitos portadores demostró diferencias estadísticamente significativas ($p<0,05$) especialmente a los tiempos iniciales de la cinética y para la mayor parte de los tejidos estudiados, excepto el riñón y la médula ósea. Por otra parte, los parámetros farmacocinéticos modelo-independiente demostraron un incremento en el área bajo la curva de niveles plasmáticos en el plasma y en ciertos tejidos como el pulmón. También se ha observado un incremento en la semivida aparente del fármaco en tejidos como bazo, hígado y pulmón y especialmente en macrófagos peritoneales y monocitos cuando se administra incorporado en eritrocitos portadores, lo que demuestra claramente el efecto de acción sostenida del fármaco incorporado en este sistema portador como se observa en la figura 19.

La alta selectividad de la zidovudina por tejidos diana cuando se administra en eritrocitos portadores se recoge claramente en la figura 20, que muestra los niveles simulados de AZT en plasma y diferentes tejidos mediante corrección de los niveles tisulares reales con la fracción de fármaco liberada de forma inmediata

desde los eritrocitos. Esta figura demuestra claramente las importantes modificaciones en la farmacocinética de AZT encapsulada en eritrocitos portadores en plasma y tejidos como riñón, pulmón, bazo y macrófagos peritoneales.

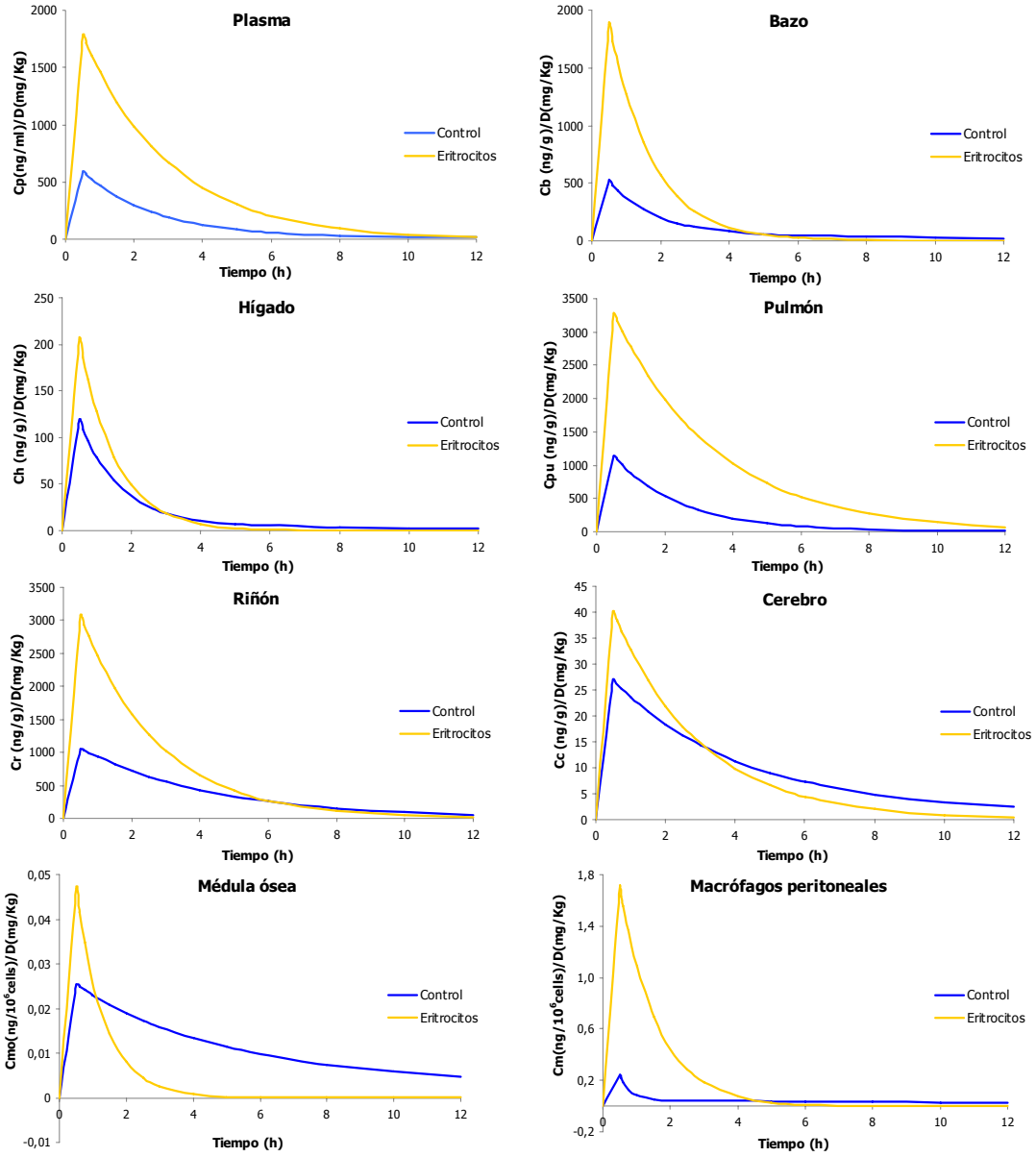

Figura 20. Simulación de las concentraciones corregidas de AZT en plasma y tejidos correspondiente a la administración de una dosis unitaria de AZT encapsulado en eritrocitos comparadas con las concentraciones obtenidas en el grupo control.

El diseño, metodología y resultados de los estudios farmacocinéticos y de biodistribución de zidovudina en ratas administrada en eritrocitos portadores han sido enviados para su publicación a la revista *The AAPS Journal*, y se encuentran recogidos en el capítulo 6 de esta memoria.

En conclusión, los eritrocitos portadores de zidovudina incrementan los niveles del fármaco en tejidos como el pulmón y el bazo y en reservorios del HIV-1 como macrófagos y monocitos. En consecuencia, este tipo de sistemas portadores puede considerarse una alternativa de futuro a otros sistemas portadores en el campo de la terapéutica antirretroviral con este y otros fármacos.

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CONCLUSIONES

1. Se han puesto a punto métodos de encapsulación de los agentes antiinfecciosos amicacina y zidovudina en eritrocitos de rata basados en la diálisis hipotónica. Los eritrocitos portadores obtenidos con este método presentaron un rendimiento de encapsulación adecuado a los objetivos del estudio, así como características morfológicas, fragilidad osmótica y parámetros hematológicos apropiados para su utilización como portadores biológicos de los fármacos estudiados.

2. Los estudios de liberación *in vitro* de los fármacos evaluados desde eritrocitos de rata demostraron una liberación sostenida, caracterizada por una rápida cesión del fármaco en los primeros minutos de la experiencia, quedando un porcentaje de fármaco encapsulado inicialmente, retenido en el interior de los eritrocitos portadores durante periodos prolongados de tiempo. Cuando se utilizó un fármaco apolar como zidovudina, la pérdida inicial de fármaco desde los eritrocitos fue muy superior a la observada para fármacos de naturaleza polar como amicacina. El tratamiento previo de eritrocitos con diamida no consiguió reducir la pérdida inicial de zidovudina.

3. El estudio de la captación *in vitro* de amicacina por macrófagos peritoneales de rata utilizando monocapas celulares demostró que los eritrocitos portadores son fagocitados por los macrófagos obteniéndose una mayor acumulacion del fármaco en las células fagocíticas.

4. Tras la administración intraperitoneal en ratas de amicacina incorporada en eritrocitos portadores, se ha observado un efecto de liberación prolongada caracterizado por un incremento en la semi-vida plasmática del antibiótico. Asimismo la biodistribución de amicacina incorporada en eritrocitos ha demostrado una mayor acumulación del fármaco en bazo e hígado y cambios moderados en la farmacocinética en pulmón, corteza renal y médula renal. La mayor acumulación de amicacina en macrofagos peritoneales *in vivo* cuando se administran eritrocitos portadores confirma los resultados obtenidos en estudios *in vitro*.

5. El estudio de los factores que condicionan la encapsulación de zidovudina en eritrocitos ha demostrado que la concentración inicial de fármaco en la bolsa de diálisis, el tiempo de diálisis y la relación de volúmenes entre el medio tamponado externo y el tampón de diálisis influyen en la concentración de fármaco encapsulado. Asimismo se ha optimizado un modelo de regresión lineal múltiple entre la concentración de fármaco encapsulada y las variables estudiadas.

6. El análisis estadístico de cinco parámetros hematológicos de los eritrocitos encapsulados con zidovudina mediante análisis inferencial y análisis de clusters ha permitido demostrar que en las condiciones de encapsulación más extremas y especialmente utilizando tiempos de diálisis iguales o superiores a 60 minutos se producen modificaciones estadísticamente significativas en parámetros como el volumen corpuscular medio (HCT), la hemoglobina corpuscular media (MCH) y la concentración de hemoglobina corpuscular media (MCHC).

7. El estudio de la fragilidad osmótica de los eritrocitos conteniendo zidovudina demostró que no existen diferencias estadísticamente significativas en la fragilidad osmótica de los eritrocitos obtenidos en las diferentes condiciones de diálisis, aunque el índice de fragilidad osmótica fue ligeramente superior en los eritrocitos tratados respecto al control.

8. La administración de zidovudina por vía intraperitoneal incorporada en eritrocitos portadores en ratas produce cambios significativos en la cinética plasmática y tisular caracterizados por un incremento en los niveles plasmáticos de zidovudina así como en los niveles titulares y en la semi-vida de la fase terminal en tejidos específicos como bazo, riñón, macrófagos peritoneales y monocitos de la médula ósea. Asimismo se ha observado una alta acumulación del fármaco en órganos del sistema retículo-endotelial como el bazo y en células fagocíticas como monocitos de la médula ósea y macrófagos.

9. Los resultados obtenidos utilizando fármacos modelo de características físico-químicas diferentes como la amicacina o la zidovudina demuestran como en ambos casos los eritrocitos constituyen un potencial sistema portador de fármacos antiinfecciosos, condicionando un efecto de liberación prolongada así como una elevada selectividad a órganos y tejidos del sistema retículo endotelial y especialmente a macrófagos residentes que constituyen un reservorio de bacterias y virus.

Salamanca, 15 de Julio de 2009

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