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DIRECCIÓN DE NUEVAS CREACIONES**

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Patente de Invención ☒

Vía Nacional ☐

Vía PCT (Fase Nacional) ☒

Patente de Modelo de Utilidad ☐

Cap. I ☒

Cap. II ☐

No. Solicitud Internacional PCT/EP2006/069643

Fecha de Presentación Internacional 13 Diciembre 2006

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

1. Documentos estudiados

Descripción:	Folios 22 a 34	25/Julio/2008
Reivindicaciones:	Folios 35 a 38	25/Julio/2008
	Folios 88 a 89	09/Julio/2012
Dibujos:	Folios 40 a 42, 46 y 47	25/Julio/2008
Prioridad:	IT MI 2006 A000122	25/Enero/2006

2. Análisis de la Prioridad

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

3. Objeto de la invención

Título de la solicitud: "Composiciones que comprenden extracto de lúpulo en aplicación liposómica para la resequedad vaginal" (modificado en el folio 81).

El problema planteado en esta solicitud consiste en la necesidad de suministrar composiciones útiles para el tratamiento de la resequedad vaginal (vaginitis atrófica) y de los trastornos relacionados con ésta, prurito, sensación de ardor, resequedad, dispareunia, y particularmente útiles para retrasar y atenuar los cambios en el tropismo fisiológico de la mucosa y los tejidos vaginal y vulvar.

Para resolver este problema técnico la solicitud en estudio proporciona el uso de lúpulo (*Humulus lupulus*) para la preparación de una especialidad medicinal, o de un dispositivo médico, o de un cosmético, o de un producto sanitario, para la aplicación

Expediente 08 77415 2do Art 45

a la región vaginal y/o vulvar.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A 61 K 36/185; A 61 P 15/02.

4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

Aunque la reivindicación 2 se encuentra encabezada como una composición, el producto se define por referencia a un uso. En consecuencia, la reivindicación 2 se considera como un uso, y por lo tanto no es patentable, de acuerdo con los Art 14 D 486.

5. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es clara porque las expresiones "excipiente liposomal" y "agentes de hidratación" definen los componentes de la invención por medio de su función y no a través de sus características estructurales. Se sugiere incluir en la reivindicación 1 los compuestos específicos que hacen parte de la invención definiéndolos por medio de su nombre IUPAC o denominación común internacional, así como sus proporciones. Los excipientes liposómicos reclamados en la reivindicación 9 pueden incluirse dentro de la reivindicación 1, pero es necesario definir los compuestos específicos que pertenecen al grupo de los fosfolípidos, esfingolípidos, glicolípidos y/o proteolípidos y que hacen parte de la invención. Adicionalmente, en las realizaciones preferidas divulgadas en la descripción sólo existe sustento para el excipiente liposomal lecitina de soya y para el agente hidratante propilenglicol.

La reivindicación 2 no es clara porque indica el uso que puede darse al producto reivindicado, el cual no es una característica técnica que defina a la invención. Se sugiere eliminarla del pliego reivindicatorio.

La expresión "se utiliza" resta claridad a la reivindicación 3 debido a que parece hacer referencia al uso de la invención. Adicionalmente, se reclama como parte de la invención un extracto de lúpulo, pero no se definen sus características esenciales. Se sugiere reemplazar la expresión "se utiliza" por una más adecuada e incluir el contenido de la reivindicación 4 dentro de la reivindicación 3.

La reivindicación 5 presenta una falsa dependencia de la reivindicación 1 ya que reclama un contenido de lúpulo entre 0.05 y 25% en peso con respecto al peso de la formulación mientras que la reivindicación 1 reclama un rango más estrecho: entre 0.1 y 15% en peso con respecto al peso de la formulación. Se sugiere eliminar la reivindicación 5.

La reivindicación 6 resta concisión al capítulo reivindicatorio ya que su contenido es redundante con lo reclamado en la reivindicación 1 y no aporta ninguna característica adicional a las ya reclamadas en reivindicaciones anteriores. Se sugiere eliminar la reivindicación 6.

Las reivindicaciones 8, 13, 15 y 17 no son claras porque las expresiones "excipiente farmacológicamente aceptable", "agentes de ionización", "antioxidantes", "agentes de quelación", "agentes de hidratación", "agentes de termoviscosidad", "descongestionantes", "agentes desinfectantes y/o antibacterianos", "perfumes",

“esencias”, “colorantes”, “polímero...efecto termoviscoso”, “disolventes farmacológicamente aceptados” definen los componentes de la invención por medio de su función y no a través de sus características estructurales. Se sugiere incluir los compuestos específicos que hacen parte de la invención definiéndolos por medio de su nombre IUPAC o denominación común internacional, así como sus proporciones dentro de la formulación. Si estos componentes son esenciales para la invención se sugiere incluirlos en la reivindicación 1.

La reivindicación 14 no es clara porque reclama un polímero pero no especifican los compuestos que hacen parte de la invención. Se sugiere incluir el contenido de la reivindicación 16 en la reivindicación 14 y especificar las proporciones de dichos componentes. Si el polímero es una característica esencial de la invención se sugiere incluirlo en la reivindicación 1. Adicionalmente, en las realizaciones preferidas divulgadas en la descripción sólo existe sustento para homopolímeros de ácido acrílico reticulado con poliéter polialqueno y para ácido hialurónico.

Descripción (Art 28 D 486)

Los términos “nipagina” y “nipasol” (ver folios 30, 32) son poco reconocidos en el campo técnico. Se sugiere definirlos por medio del nombre IUPAC.

Se requiere al solicitante hacer las aclaraciones necesarias.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad (Enero 25 de 2006) y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D2	WO02/076410	Cosmetic and dermatological preparations comprising a content of a hop extract or hop-malt extract and the utilization of a hop extract or hop-malt extract for producing cosmetic and dermatological preparations for reducing the sebum contents of the skin	03/Octubre/2002
D3	Pavelić Z, Skalko-Basnet N, Jalsenjak International journal of pharmaceutics. 2005;301(1-2):140-8	Characterisation and in vitro evaluation of bioadhesive liposome gels for local therapy of vaginitis.	15/Julio/2005

D2 http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&ocale=en_gb&FT=D&date=20021003&CC=WO&NR=02076410A2&KC=A2 divulga (Pág. 1, líneas 13 a 14; Pág. 24, línea 26; Reivindicaciones 6 y 7) extractos de lúpulo para la producción de preparaciones tópicas en donde el porcentaje de extracto de lúpulo puede estar entre 0.01 y 20%, preferiblemente entre 0.02 y 5.0%.

D3 <http://www.ncbi.nlm.nih.gov/pubmed?term=Int%20J%20Pharm.%202005%20Sep%2014%3B301%281-2%29%3A140-8> divulga (Abstract; Pág. 141, columna 2, párrafo 1) una composición de liberación vaginal para el tratamiento de la vaginitis, que consiste en un gel bioadhesivo que contiene liposomas formados por los fosfolípidos fosfatidilcolina de huevo (EPC) y fosfatidilglicerol sódico de huevo (EPG).

7. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en una composición caracterizada por un contenido de lúpulo entre 0.1 y 15% en peso, con respecto al peso de la formulación, un excipiente liposomal entre 0.01 y 15% en peso, con respecto al peso de la formulación y agentes de hidratación seleccionados de entre alcoholes, glicoles polihidroxi­latados, mucopolisacáridos, ácido láctico o sus sales, ácido 2-pirrolidona-carboxílico o sus sales, aceites vegetales o minerales, siliconas y derivados.

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

Características esenciales	D2	D3
Una composición caracterizada por un contenido de:	Una composición caracterizada por:	Una composición caracterizada por:
Lúpulo entre 0.1 y 15% en peso, con respecto al peso de la formulación	Extracto de lúpulo entre 0.01 y 20%	No se menciona
Un excipiente liposomal entre 0.01 y 15% en peso, con respecto al peso de la formulación	No se menciona	Excipientes liposomales como fosfatidilcolina de huevo (EPC), fosfatidilglicerol sódico de huevo (EPG) en una concentración de, por ejemplo, 0.0263% en peso con respecto al peso de la formulación
Agentes de hidratación seleccionados de entre alcoholes, glicoles polihidroxi­latados, mucopolisacáridos, ácido láctico o sus sales, ácido 2-pirrolidona-carboxílico o sus sales, aceites vegetales o minerales, siliconas y derivados	Etanol, isopropanol, etilenglicol, propilenglicol, ácido hialurónico, ácido láctico, aceites vegetales como aceite de ricino, siliconas y sus derivados.	Etanol, propilenglicol.

Los documentos D2 y D3 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D2 divulga extractos de lúpulo para la producción de preparaciones tópicas para la reducción de la producción sebácea de la piel (Pág. 1, líneas 13 a 14). En una preparación cosmética el porcentaje de extracto de lúpulo puede estar entre 0.01 y 20% (Reivindicación 6; Pág. 24, línea 26); preferiblemente entre 0.02 y 5.0% (Reivindicación 7). Los principios activos se pueden encapsular usando ceras fundidas para formar nanopartículas sólidas de lípidos o usando polímeros como polimetacrilatos reticulados (Pág. 7, líneas 9 a 16). Los ingredientes activos se pueden incorporar en formulaciones cosméticas (Pág. 7, líneas 23 a 26). A las composiciones se pueden adicionar antioxidantes, agentes quelantes (Pág. 8, líneas 11 a 35; Pág. 9, líneas 1 a 5), perfumes, colorantes, antibacterianos (Pág. 10, líneas 1 a 7), entre otros. D2 también divulga que la composición puede contener aceite de ricino (Pág. 12, líneas 22-23), etanol, isopropanol, etilenglicol, propilenglicol (Pág. 14, líneas 20-24), ácido hialurónico (Pág. 14, línea 28), ácido láctico (0041), aceites vegetales como aceite de ricino, siliconas y sus derivados (Pág. 14, líneas 7-10).

D3 divulga una composición de liberación vaginal para el tratamiento de la vaginitis,

que consiste en un gel bioadhesivo que contiene liposomas. Los liposomas contienen los principios activos clotrimazol y metronidazol (Abstract). D3 da a conocer que los liposomas están compuestos por los fosfolípidos fosfatidilcolina de huevo (EPC) y fosfatidilglicerol sódico de huevo (EPG) y son preparados por los métodos de proliposoma y de dilución en poliol. Una vez preparados, los liposomas se incorporan a un gel de un homopolímero de ácido acrílico reticulado con poliéter polialqueno (Carbopol®) (Pág. 141, columna 2, párrafo 1). La concentración de los liposomas en el gel es del 10% p/p (Pág. 142, columna 2, párrafo 3) y la concentración de los fosfolípidos en la preparación de liposomas es de 26.3% p/p (200mg de EPC+EPG en 760 mg de solución) (Pág. 142, columna 1, párrafo 2); de esta forma la concentración de fosfolípidos es de 0.0263% p/p con respecto al peso de la formulación. D3 también divulga la adición de etanol y propilenglicol a la formulación (Pág. 142, columna 1, párrafos 2 y 3).

La diferencia entre la invención, definida en la reivindicación 1 y la composición de D2 consiste en que contiene un excipiente liposomal entre 0.01 y 15% en peso con respecto al peso de la formulación.

El efecto que logra el incluir el excipiente liposomal es que se obtiene una alternativa para incorporar el extracto de lúpulo en una formulación en gel.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar la composición conocida en D2 para diseñar una composición alternativa de extracto de lúpulo para administración vaginal".

Sin embargo, la adición de excipientes liposómicos para elaborar formulaciones farmacéuticas de administración vaginal, ya está divulgada en el documento D3 que se refiere a una composición de aplicación vaginal para el tratamiento de la vaginitis, que consiste en un gel bioadhesivo que contiene liposomas formados por los fosfolípidos fosfatidilcolina de huevo (EPC) y fosfatidilglicerol sódico de huevo (EPG) (Abstract; Pág. 141, columna 2, párrafo 1). Adicionalmente, D3 enseña que los liposomas se han usado ampliamente como portadores de fármacos en el tratamiento tópico de enfermedades ya que son capaces de incorporar fármacos hidrofílicos e hidrofóbicos para aumentar la concentración de fármaco en el sitio de administración y reducir las incompatibilidades. Como los liposomas pueden hacer que el fármaco se libere de forma controlada, son una opción que puede considerarse en la aplicación vaginal. D3 también divulga que es lógico escoger un gel de polímeros derivados de ácido acrílico reticulado como vehículo para la incorporación de liposomas porque se ha demostrado que son ampliamente estables en este medio y adicionalmente el polímero tiene excelentes propiedades bioadhesivas en la superficies mucosas (Pág. 141, columna 1, párrafo 3)

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría un excipiente liposómico de D3 en la composición de D2 para llegar así al objeto de la reivindicación 1 de la solicitud; por lo cual la reivindicación 1 se considera obvia.

Puesto que las reivindicaciones dependientes 2 a 17 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:

Las reivindicaciones 1 a 17 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

8. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D2	WO02/076410	1 – 17	Nivel Inventivo
D3	Pavelić Z, Skalko-Basnet N, Jalsenjak I. International journal of pharmaceutics. 2005;301(1-2):140-8	1 – 17	Nivel Inventivo

9. Respuesta a los argumentos del señor solicitante

Debido a que el solicitante presenta un nuevo capítulo reivindicatorio modificando la materia reclamada de un uso a una composición, los documentos D1 y D2 no afectan la novedad de la invención. Por tal motivo, se llevó a cabo una nueva búsqueda para realizar el examen de patentabilidad, encontrándose que los documentos D2 y D3 afectan el requisito de nivel inventivo.

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

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


JOSÉ LUIS SALAZAR LÓPEZ

Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha

~~28 AGO. 2012~~

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Characterisation and in vitro evaluation of bioadhesive liposome gels for local therapy of vaginitis

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Abstract

The purpose of this study was to design and evaluate a new vaginal delivery system for the local treatment of vaginitis. Liposomes containing two commonly applied drugs in the treatment of vaginal infections, namely clotrimazole and metronidazole, were prepared by the proliposome and the polyol dilution methods. Both types of liposomes were characterised and compared for particle size, polydispersity, entrapment efficiency, and tested for in vitro stability in media that mimic human vaginal conditions (buffer, pH 4.5, and vaginal fluid simulant). To achieve application viscosity and to further improve their stability, liposomes containing drugs were incorporated in a bioadhesive gel made of Carbopol® 974P NF resin. In vitro release studies have demonstrated that even after 24 h of incubation in vaginal fluid simulant (at 37 °C) more than 30% of the originally entrapped clotrimazole (or 50% of metronidazole) was still retained in the gel. Storage stability studies have proved the ability of Carbopol® 974P NF gel to preserve original size distributions of incorporated liposomes. All the performed experiments confirm the applicability of bioadhesive liposome gels as a novel delivery system for local therapy of vaginal infections.

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Keywords: Liposomes; Clotrimazole; Metronidazole; Bioadhesive gel; Stability; Vaginal infections

1. Introduction

Vaginitis is one of the most frequent gynaecological diseases. Common causes of vaginitis are candidiasis and bacterial vaginosis due to infection with mixed

bacterial flora. *Candida* is an opportunistic pathogen, causing infection when host defences are impaired or the normal microbial balance is upset. It has been estimated that 50–75% of women will experience candidal vaginitis at some time in their lives (Kükner et al., 1996). Bacterial vaginosis is synonymous with Gardnerella-associated vaginitis and anaerobic vaginosis. It occurs when vaginal concentration of anaerobic bacteria reach 100–100,000 times that in

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normal vagina, in association with suppression of some components of the normal vaginal flora, such as *Lactobacillus species* (Fischbach et al., 1993). For the treatment of vaginitis, local antimicrobial administration of clotrimazole (for *Candida* infections) or metronidazole (bacterial vaginosis), has been favoured due to the numerous side effects, toxicity, and teratogenic potential of the systemically applied drugs (Bouckaert et al., 1995; Kükner et al., 1996).

To achieve desirable therapeutic effect, vaginal delivery systems for antimicrobial agents need to reside at the sites of infection for a prolonged period. Applied by the vaginal route, clotrimazole and metronidazole have a residence time of the delivery system mostly insufficient to provide adequate therapeutic effect (Fischbach et al., 1993; Hillier et al., 1993; Livengood et al., 1994). Hence, there is a need to develop effective drug delivery systems that should prolong the contact of the drug with a mucosal surface and enable sustained release of incorporated drug. Several novel carrier systems were suggested to be appropriate for vaginal drug delivery: bioadhesive tablets (Bouckaert et al., 1995), polycarboxylic hydrogels (Knuth et al., 1993; Brown et al., 1997), microspheres (Richardson et al., 1996), and liposomes (Foldvari and Moreland, 1997; Jain et al., 1997).

Liposomes have been widely used as drug carriers in topical treatments of diseases, especially in dermatology. They are capable to incorporate a variety of hydrophilic and hydrophobic drugs, to enhance the accumulation of drug at the administration site and to reduce side effects and incompatibilities (Hope and Kitson, 1993). Since liposomes can provide sustained and/or controlled release of entrapped drug, they are considered to be applied vaginally, too (Jain et al., 1997). However, the major limitation of using liposomes topically and vaginally, is the liquid nature of preparation. That can be overcome by their incorporation in an adequate vehicle where original structure of vesicles is preserved (Škalko et al., 1998). It has already been shown that liposomes are fairly compatible with gels made from polymers derived from crosslinked poly(acrylic acid), such as Carbopol® resins (Pavelić et al., 1999). Moreover, some Carbopol® have proved excellent bioadhesive properties on the mucosal surface (Dittgen et al., 1997). Therefore, it seemed logical to choose gels prepared from those resins as a vehicle

for the incorporation of liposomes destined for vaginal delivery.

A previous study has suggested application of liposomes containing antimicrobial drugs for the local therapy of vaginitis (Pavelić et al., 1999). Continuing that research, here, we report about design and in vitro evaluation of a bioadhesive liposome gels containing clotrimazole and metronidazole. Liposomes composed of egg phosphatidylcholine (EPC) and egg phosphatidylglycerol-sodium (EPG) in the molar ratio 9:1, were prepared by the proliposome and the polyol dilution methods and incorporated in the bioadhesive Carbopol® gel. To be closer to an actual in vivo application in women, liposomal gels were tested in media that simulate human vaginal conditions.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC), egg phosphatidylglycerol-sodium (EPG), and Carbopol® 974P NF were generous gifts from Lipoid GmbH (Ludwigshafen, Germany) and BFGoodrich (Brussels, Belgium), respectively. Clotrimazole was a gift from Bayer Pharma (Ljubljana, Slovenia). Metronidazole was the product of Fischer (Zurich, Switzerland). Bovine serum albumin and lactic acid were purchased from Sigma Chemicals (Deisenhofen, Germany).

Phosphate buffer, pH 7.4 was composed of 8 g/l NaCl, 0.19 g/l KH_2PO_4 , and 2.38 g/l Na_2HPO_4 . Phosphate buffer, pH 4.5 was made of 13.61 g/l KH_2PO_4 in demineralised water.

Vaginal fluid simulant (VFS) was prepared from 3.51 g/l NaCl, 1.40 g/l KOH, 0.222 g/l $\text{Ca}(\text{OH})_2$, 0.018 g/l bovine serum albumin, 2 g/l lactic acid, 1 g/l acetic acid, 0.16 g/l glycerol, 0.4 g/l urea, 5 g/l glucose. pH of the mixture was adjusted to 4.5 using 0.1 M HCl (Owen and Katz, 1999).

All chemicals and solvents used in the experiments were of analytical grade.

2.2. Preparation of liposomes

Liposomes containing clotrimazole or metronidazole were prepared by two methods: the proliposome (Perrett et al., 1991), and the polyol dilution

method (Kikuchi et al., 1994). Concentration of lipids used was the same for all preparations (26 mM, total lipid).

2.2.1. Proliposome method (procedure)

EPC (180 mg), EPG (20 mg), clotrimazole (20 mg) were mixed with warm ethanol (160 mg) and the phosphate buffer, pH 7.4 (400 mg) to achieve an initial proliposome mixture. The mixture was stirred for 2 min at 60 °C, cooled to room temperature and then converted to a liposome suspension by drop-wise addition of 10 ml of the buffer, pH 7.4. During the last stage of this procedure, the suspension was stirred continuously (600 rpm) for 60 min.

2.2.2. Polyol dilution method (procedure)

EPC, EPG (molar ratio 9:1), and clotrimazole (20 mg) were dissolved in propylene glycol (500 mg) at 60 °C, and 500 mg of phosphate buffer, pH 7.4 (previously warmed to the same temperature) was poured into the lipid-drug-polyol solution. The suspension was diluted by the addition of a buffer, pH 7.4 (10 ml) during continuous magnet stirring (600 rpm) at 60 °C for 45 min and then cooled to room temperature.

Liposomes containing metronidazole (50 mg taken into preparation, both methods) were prepared under exactly the same conditions, as above.

Finally, all liposome suspensions were extruded three times through 0.4 µm polycarbonate membrane filters (LiposoFast, Avestin, Ottawa, Canada).

Lipid concentration was determined by quantification of inorganic phosphate using the method of Bartlett (Bartlett, 1959).

2.3. Entrapment efficiency determination

The entrapment efficiencies were examined to compare the encapsulation of drug in liposomes prepared by different methods. Untrapped (free) drug was separated from liposomal drug by gel chromatography on Sepharose CL 4B (Pharmacia, Sweden) column using phosphate buffer, pH 7.4 for elution. The concentrations of the drugs (both in liposomes and free) were determined in all collected fractions spectrophotometrically, as previously described (Pavelić et al., 1999).

Recovery of drug was determined for all samples and was between 89.6 and 94.7% of the amount taken into preparations.

2.4. Vesicle size determination

Morphology, size distributions, and mean diameters of the liposomes were determined by image analysis microscopy technique. Morphology and size distribution of vesicles (based on the number of particles) were determined in an Olympus BH-2 microscope equipped with a computer-controlled image analysis system (Optomax V, Cambridge). In all the experiments, approximately 10,000 liposomes were examined (Škalko-Basnet et al., 2000). Also, mean diameters, as well as polydispersity index and zeta potential of the liposomes, were determined by photon correlation spectroscopy (PCS) (Zetamaster, Malvern Instruments, Malvern, UK) 24 h after their preparation (Pavelić et al., 2001).

2.5. Preparation of gel

As a vehicle for incorporation of liposomes for vaginal delivery, bioadhesive gel was made. Carbopol® 974P NF (1 g) was dispersed in demineralised water (88 g) by stirring at 800 rpm for 60 min. Then propylene glycol (10 g) was added and the mixture was neutralised by drop-wise addition of 10% NaOH. Mixing was continued until a transparent gel appeared, whereas the amount of base was adjusted to achieve a gel with pH 5.5 (Pavelić et al., 2001).

2.6. Incorporation of liposomes into the gel

Liposomes containing clotrimazole, or metronidazole (previously separated from the untrapped drug) were mixed into the 1% (w/w) Carbopol® gel by an electrical mixer (25 rpm, 2 min) with the concentration of liposomes in gel being 10% (w/w, liposome suspension/total). Control gels (10% (w/w)) were made under the same conditions. Instead of liposomes those samples contained free clotrimazole or metronidazole (Škalko et al., 1998).

2.7. In vitro stability studies

Liposome preparations (both suspensions and gels) were tested for in vitro stability in the phosphate buffer, pH 4.5. To be closer to human conditions, the same experiments were performed in the vaginal fluid simulant (VFS), pH 4.5.

2.7.1. Liposome suspensions

Liposomes (1 ml), separated from untrapped drug, were dispersed in the buffer, pH 4.5 (5 ml) and incubated at 37 °C using a water bath. Samples were taken at certain time intervals (1, 2, 4, 6, and 24 h), separated from the released drug and the amount of drug determined spectrophotometrically (Pavelić et al., 1999).

2.7.2. Liposomal gels

Procedure modified by Peschka et al. (1998) was applied: samples of gel with incorporated liposomes containing clotrimazole or metronidazole (3 g) were put in glass vials and separated from the receptor solution (phosphate buffer pH 4.5, or VFS) by a layer of 2% agarose gel. The vials were incubated at 37 °C and the receptor solution was completely removed at certain time intervals (1, 2, 4, 6, and 24 h). The amount of the released drug was determined spectrophotometrically before and after the addition of methanol.

Simultaneously and under the same conditions, control experiments (Carbopol® gels with free clotrimazole or metronidazole) were done.

2.8. Storage stability study

Samples of gel with liposomes containing drug (clotrimazole or metronidazole) were stored at 20 and 40 °C during 4 weeks. The physical stability of the incorporated liposomes was determined by image analysis microscopy technique (as described above). In all the experiments, approximately 5000 liposomes were examined.

3. Results and discussion

3.1. Characterisation of liposomes

The desirable local therapeutic effect of liposomes as drug carriers can be achieved if they are loaded with a sufficient amount of active compound. For that reason liposomes with clotrimazole and metronidazole were prepared by two methods and compared for entrapment efficiency, vesicle size and polydispersity (Table 1). Regardless of the preparation procedure used and the encapsulated drug, mean diameters of liposomes were between 270 and 320 nm. There was no statistically significant difference (*t*-test, *p* > 0.05) between average diameters of liposomes detected by different measuring techniques; image analysis or PCS. However, liposomes prepared by the polyol dilution method (PD liposomes) were of a more homogenous size distribution (data not shown) than those prepared by the proliposome method (proliposomes). This was probably a consequence of the preparation procedure based on the preparing of concentrated proliposome mixture, which was then converted to stable liposome dispersion by the dilution of the excess aqueous phase (Perrett et al., 1991). Therefore, even after extrusion was performed three times through 400 nm pore size membrane filters, a small amount of the residual proliposome mixture still remained in the final liposome dispersion affecting the polydispersity index (about 0.300, regardless of incorporated drug) in comparison to PD liposomes (about 0.240, respectively). Because the same lipid composition was used for all liposome preparations, zeta potential values were similar (Table 1). Considering the entrapments of drugs, rather high entrapment was

Table 1
Characterisation of liposomes

Parameter	Liposomes with clotrimazole		Liposomes with metronidazole	
	Proliposomes	PD liposomes	Proliposomes	PD liposomes
Mean diameter (nm)	277.9 ± 3.0 ^a 310.1 ± 4.7 ^b	293.6 ± 6.1 ^a 315.8 ± 7.5 ^b	298.7 ± 5.8 ^a 320.1 ± 6.2 ^b	305.1 ± 3.1 ^a 331.4 ± 7.2 ^b
Polydispersity index	0.307 ± 0.040	0.248 ± 0.021	0.295 ± 0.034	0.240 ± 0.054
Zeta potential (mV)	−56.5 ± 4.2	−57.8 ± 3.3	−54.6 ± 6.0	−55.7 ± 2.1
Entrapment efficiency (%)	71.2 ± 5.9	64.3 ± 8.9	7.3 ± 1.2	5.5 ± 0.9

Liposomes containing clotrimazole or metronidazole were prepared by the proliposome and the polyol dilution method and extruded three times through polycarbonate membrane filters, pore size 400 nm. Mean diameters were determined by PCS^a as well as by an image analysis technique.

^bEntrapment of drug in liposomes was determined as described earlier. The values denote the mean ± S.D. (*n* = 3).

observed with lipophilic drug clotrimazole (64–71%) (Table 1). That could be explained by its affinity to be incorporated in the phospholipid bilayers of liposomes. However, both preparation methods resulted in low entrapment efficiencies for metronidazole, a drug known for its low solubility in both hydrophilic and lipophilic media ($\log P=0.75$) (Mafhouz and Hassan, 2001). In order to confirm possible interactions of the drug with liposome membranes further studies based on differential scanning calorimetry can be recommended.

3.2. In vitro stability and release studies

In order to develop a liposomal drug carrier system for localised and sustained vaginal delivery, it was necessary to check the stability of liposomes in conditions simulating vaginal environment. Since, healthy human vaginal mucous is characterised by pH ranging between 4.0 and 5.0 (Caillouette et al., 1997) experiments were performed in the pH 4.5 phosphate buffer (Pavelić et al., 1998). A variety of compounds from the vaginal fluid can influence the flow, retention, drug delivery kinetics, and bioactivity of therapeutic formulations applied vaginally (Owen and Katz, 1999). Therefore, in vitro stability of liposomes was examined in the VFS, too. As can be seen in Fig. 1, liposomes (prepared by both methods and containing different drugs) were most stable at pH 7.4 (control). However, at pH 4.5 a fast release of the originally entrapped clotrimazole was observed within first hour of incubation at 37 °C and slower in the following 23 h (Fig. 1A). So even after 24 h, more than 55% of the drug still remained in the liposomes, regardless of the preparation procedure used. When experiments were carried out in VFS, negligible lower amount of originally entrapped clotrimazole was determined in liposomes (in comparison to results at pH 4.5), thus confirming predominant effect of pH on the stability of liposomes (Fig. 1A). Similar patterns in the pH 4.5 and VFS were observed with liposomes containing metronidazole (Fig. 1B). There was no found statistically significant difference (*t*-test, $p > 0.05$) between stability of liposomes in the buffer, pH 4.5, and VFS for both preparations.

In previous experiments with hydrophilic model substances of different molecular weights: calcein Mw 622.5 (Pavelić et al., 2001), FITC-dextran Mw 4400 and FITC-dextran 21,200 (Pavelić et al., 2004), in the

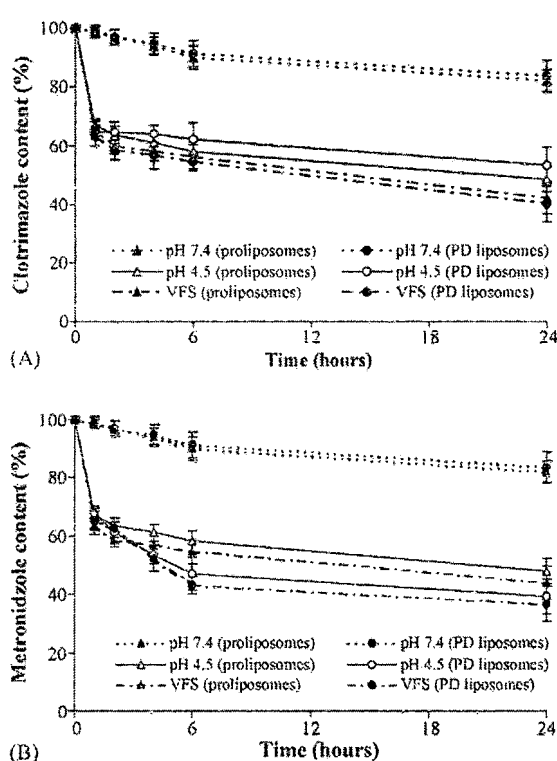


Fig. 1. In vitro stability of liposomes with clotrimazole (A) and metronidazole (B). Liposome suspensions, free from the unentrapped drug, were incubated in different testing media at 37 °C. Samples were taken at certain time intervals and the concentration of the drug still present in liposomes was determined spectrophotometrically. Indicated values are the means of three experiments ($\pm$ S.D.).

buffer pH 4.5, liposomes prepared by both methods could retain a higher amount of originally entrapped marker (e.g. more than 60% calcein was remained in PD liposomes after 24 h, or even more than 80% of FITC-dextran 21,200, respectively). Comparison of those findings with the results obtained with clotrimazole and metronidazole, demonstrate a better stability of water-soluble compounds. That could be explained by the hydrophilic nature of calcein at neutral pH in liposomes, whereby membrane penetration is very slow and liposomes could retain a higher amount of the originally entrapped calcein. In the case of FITC-dextran, an even better stability in the buffer pH 4.5 than in study with calcein, is probably a consequence of steric effects due to the higher molecular weight of encapsulated compounds.

To be closer to application of liposomes in humans, an appropriate viscosity of liposomal preparations is

necessary. This can be achieved by their incorporation in a vehicle suitable for vaginal self-administration, such as a gel. Because a prolonged retention on the mucous is often required for the desired therapeutic effect, research efforts have been made on using hydrophilic polymers with bioadhesive characteristics to improve drug delivery via vagina (Knuth et al., 1993). It has already been proven that liposomes are compatible with polymers derived from polyacrylic acid, such as Carbopol® resins (Škalko et al., 1998; Pavelić et al., 2001). Since Carbopol® 974P NF is known for its excellent bioadhesivity on the mucous, it seemed reasonable to choose that resin to make a gel-like vehicle for the incorporation of liposomes.

In this study, proliposomes containing clotrimazole or metronidazole were mixed into 1% Carbopol® 974P NF gel and tested for in vitro release of the entrapped drugs. As described earlier (Pavelić et al., 2001), a modified method by Peschka et al. (1998) was applied to follow the release of clotrimazole (or metronidazole) from liposomes incorporated in the bioadhesive gel. The porosity of the 2% agarose matrix permitted intact liposomes and released (free) drug to diffuse through matrix into the receptor solution (buffer, pH 4.5 or VFS). The amount of the drug released from the gel was determined spectrophotometrically, before and after the addition of methanol to the supernatant over the agarose matrix.

Results presented in Tables 2 and 3 confirm a slower release of both drugs from liposomes after their incor-

poration in the bioadhesive gel. Moreover, incorporation of liposomes in the gel further improved their stability in both tested media. After 24 h of incubation at 37 °C in the buffer pH 4.5, more than 70% of the originally entrapped clotrimazole was still retained in liposomes incorporated in the gel, in comparison to control (2%). There were no found statistically significant differences (*t*-test, $p > 0.05$) between values obtained at pH 4.5 and VFS (Table 2). When the gel with liposomes containing metronidazole was tested, a faster release of the drug was observed both in buffer pH 4.5 and VFS (Table 3).

The model used to study the release of drugs from liposomes incorporated in gel gave valuable information about intact liposomes released from the gel through agarose matrix into the receptor media. That value (intact liposomes) was calculated as a difference between total drug (after addition of methanol) and free drug (before addition of methanol) to the supernatant over the gel. As presented in Tables 2 and 3, the percentage of diffused intact liposomes was higher in the buffer pH 4.5 than in VFS (*t*-test, $p > 0.05$), indicating less stability of liposomes in the presence of vaginal fluid components. So after 24 h of incubation in the buffer pH 4.5, about 56% of the intact liposomes with clotrimazole were released, while 34% were released when experiments were performed in VFS. However, liposomes with metronidazole (Table 3) were less stable than liposomes containing clotrimazole (*t*-test, $p > 0.05$). After 24 h, only 9% of intact lipo-

Table 2
In vitro release of clotrimazole, entrapped in liposomes, incorporated in the gel

Time (h)	Free clotrimazole (%)	Total clotrimazole (%)	Intact liposomes (%)	Control (%)
1	6.86 ± 1.93 ^a	15.85 ± 4.07 ^a	8.99 ± 4.54 ^a	15.28 ± 2.68
	9.05 ± 5.82 ^b	19.16 ± 3.03 ^b	10.11 ± 6.40 ^b	
2	10.18 ± 2.31 ^a	28.18 ± 11.02 ^a	18.00 ± 10.55 ^a	26.48 ± 5.20
	14.38 ± 7.48 ^b	28.63 ± 4.62 ^b	14.25 ± 8.63 ^b	
4	14.52 ± 2.48 ^a	42.26 ± 11.81 ^a	27.74 ± 12.25 ^a	38.07 ± 6.89
	19.84 ± 8.80 ^b	38.76 ± 5.04 ^b	18.91 ± 8.88 ^b	
6	17.64 ± 3.00 ^a	56.60 ± 17.13 ^a	38.96 ± 17.03 ^a	50.94 ± 8.60
	24.88 ± 9.91 ^b	48.89 ± 6.53 ^b	24.01 ± 10.43 ^b	
24	24.58 ± 2.06 ^a	80.94 ± 24.47 ^a	56.36 ± 24.46 ^a	98.14 ± 9.84
	33.26 ± 11.95 ^b	68.10 ± 9.67 ^b	34.84 ± 12.41 ^b	

Samples of gel containing proliposomes were incubated in the buffer, pH 4.5 (for details see Section 2). The amount of clotrimazole was determined in the release media before and after disruption of liposomes by the addition of methanol. Control experiments (gel containing free clotrimazole, without liposomes) were performed simultaneously. Indicated values are the means of three experiments (±S.D.).

^a Buffer, pH 4.5.

^b VFS, pH 4.5.

Table 3
In vitro release of metronidazole, entrapped in liposomes, incorporated in the gel

Time (h)	Free metronidazole (%)	Total metronidazole (%)	Intact liposomes (%)	Control (%)
1	4.69 ± 0.46 ^a 5.02 ± 0.19 ^b	6.94 ± 0.87 ^a 5.36 ± 0.50 ^b	2.25 ± 0.46 ^a 0.34 ± 0.30 ^b	6.93 ± 3.43
2	9.67 ± 1.32 ^a 10.21 ± 0.15 ^b	13.82 ± 2.36 ^a 11.03 ± 0.67 ^b	4.15 ± 1.07 ^a 1.18 ± 0.49 ^b	13.37 ± 3.89
4	16.67 ± 2.80 ^a 17.39 ± 0.46 ^b	22.20 ± 3.95 ^a 19.11 ± 1.80 ^b	5.53 ± 1.92 ^a 1.72 ± 1.70 ^b	28.36 ± 6.11
6	23.10 ± 4.53 ^a 23.71 ± 0.62 ^b	30.34 ± 5.40 ^a 26.54 ± 2.41 ^b	7.24 ± 2.42 ^a 2.82 ± 2.70 ^b	37.64 ± 7.23
24	32.39 ± 10.65 ^a 42.24 ± 2.36 ^b	46.24 ± 12.42 ^a 51.25 ± 3.59 ^b	13.85 ± 3.09 ^a 9.01 ± 3.97 ^b	88.41 ± 7.21

Samples of gel containing proliposomes were incubated in the buffer, pH 4.5 (for details see Section 2). The amount of metronidazole was determined in the release media before and after disruption of liposomes by the addition of methanol. Control experiments (gel containing free metronidazole, without liposomes) were performed simultaneously. Indicated values are the means of three experiments (±S.D.).

^a Buffer, pH 4.5.

^b VFS, pH 4.5.

somes were detected in the presence of vaginal fluid components.

When the amount of retained clotrimazole (or metronidazole) in gel is plotted against the square root of time, a linear correlation according to Higuchi equation (Higuchi, 1961) is obtained, thus showing matrix-controlled diffusion of the released drug (Fig. 2).

In studies with hydrophilic compounds (Pavelić et al., 2001, 2004), after 24 h of incubation in the buffer pH 4.5 only 16% of the originally encapsulated calcein, or 12% of FITC-dextran 4400, or 8% of FITC-dextran 21200 were released from the Carbopol® gel. Those findings were expected because of the inverse correlation between the release rate and the molecular weight of the encapsulated marker. In the present research, faster release of metronidazole from liposomes incorporated in gel was probably due to the physico-chemical characteristics of the drug (Mafhouz and Hassan, 2001). Since pH of gel was 5.5 and investigations were performed in media of lower pH (pH 4.5), due to solubility of drug in acid, these results seem to be quite reasonable.

Regarding the physical properties of a vehicle for incorporation of liposomes, it has been confirmed that Carbopol® 974P NH gel offers adequate pH value and rheological properties (Pavelić et al., 2001). Due to the well-known loss of viscosity caused by sodium ions (Dittgen et al., 1997) from buffer in which liposomes were made, original gel was made in the concentration of 1% (w/w) and after addition of 10% liposomes,

suitable application viscosity was achieved (Pavelić et al., 2001). In order to provide a stable vehicle for vaginal administration in which liposomes are distributed uniformly and their structure is preserved, a storage stability study was performed. Liposomal gels with clotri-

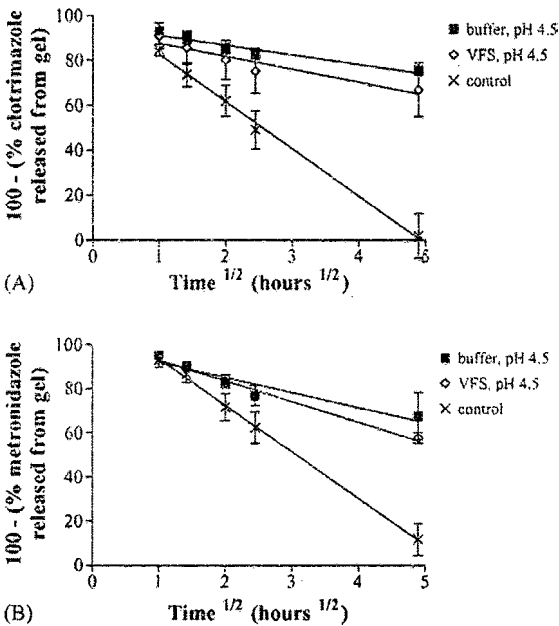


Fig. 2. Cumulative release of clotrimazole (A) and metronidazole (B) from proliposomes incorporated in bioadhesive gel in different media. The data are plotted according to Higuchi Eq. (22). Indicated values are the means of three experiments (±S.D.).

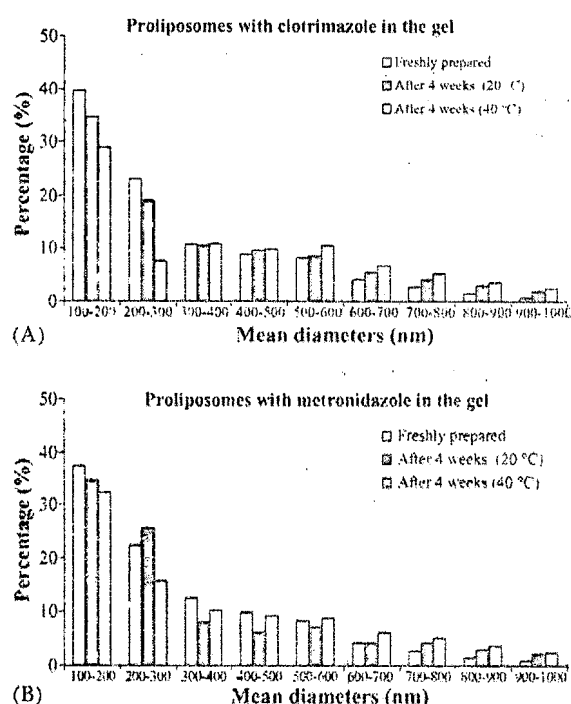


Fig. 3. Size distributions of liposomes incorporated in gel and stored for 4 weeks at 20 and 40 °C. Proliposomes containing clotrimazole (A) or metronidazole (B) were incorporated in 1% Carbopol® 974P NF gel and tested for storage stability by image analysis microscopy (for details see the text). Mean diameters of liposomes were: 310 nm (A) or 320 nm (B) immediately after incorporation in a vehicle, 373 nm (A) or 378 nm (B) after 4 weeks at 20 °C, and 414 nm (A) or 426 nm (B) after 4 weeks at 40 °C, respectively.

mazole and metronidazole were kept for 4 weeks at 20 and 40 °C (stress testing). During those experiments, the size distribution and mean diameter of the incorporated liposomes were determined. Results presented in Fig. 3 demonstrate that after 4 weeks of storage at stress conditions (40 °C), the mean diameter changed from 310 to 414 nm, but the distribution stayed statistically similar (Fig. 3A). A similar behaviour was obtained with preparation containing metronidazole (Fig. 3B).

4. Conclusions

Proliposome and polyol dilution methods yield similar entrapment efficiency for both clotrimazole and metronidazole. Both types of liposomes were similarly stable in the buffer pH 4.5, chosen to simulate vaginal pH, as well as in the VFS. Incorporation of liposomes

containing clotrimazole (or metronidazole) in a bioadhesive gel improved their stability in media that mimic vaginal environment and slowed down the release of the drugs, confirming possible application of liposomes containing antimicrobial drugs as a novel delivery system for local treatment of vaginitis.

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References

- Bartlett, G.R., 1959. Phosphorous assay in column chromatography. *J. Biol. Chem.* 234, 466–468.
- Bouckaert, S., Temmerman, M., Voorspoels, J., Van Kets, H., Remon, J.P., Dhont, M., 1995. Preliminary efficacy study of a bioadhesive vaginal metronidazole tablet in the treatment of bacterial vaginosis. *J. Pharm. Pharmacol.* 47, 970–971.
- Brown, J., Hooper, G., Kenyon, C.J., Haines, S., Burt, J., Humphries, J.M., Newman, S.P., Davis, S.S., Sparrow, R.A., Wilding, I.R., 1997. Spreading and retention of vaginal formulations in postmenopausal women as assessed by gamma scintigraphy. *Pharm. Res.* 14, 1073–1078.
- Caillouette, J.C., Sharp, C.F., Zimmerman, G.J., Roy, S., 1997. Vaginal pH as a marker for bacterial pathogens and menopausal status. *Am. J. Obstet. Gynecol.* 176, 1270–1277.
- Ditgen, M., Durrani, M., Lehmann, K., 1997. Acrylic polymers: a review of pharmaceutical applications. *STP Pharma Sci.* 7, 403–437.
- Fischbach, F., Petersen, E.E., Weissenbacher, J.M., Hosmann, J., Mayer, H., 1993. Efficacy of clindamycin vaginal cream versus oral metronidazole in the treatment of bacterial vaginosis. *Obstet. Gynecol.* 82, 405–410.
- Foldvari, M., Moreland, A., 1997. Clinical observations with topical liposome-encapsulated interferon α for the treatment of genital papilloma virus infections. *J. Liposome Res.* 7, 115–126.
- Higuchi, T., 1961. Rate of release of medicaments from ointment bases containing drugs in suspension. *J. Pharm. Sci.* 50, 874–875.
- Hillier, S.L., Lipinski, C., Briselden, A.M., Eschenbach, D.A., 1993. Efficacy of intravaginal 0.75% metronidazole gel for the treatment of bacterial vaginosis. *Obstet. Gynecol.* 81, 963–967.
- Hope, M.J., Kitson, C.N., 1993. Liposomes: a perspective for dermatologists. *Dermatol. Clin.* 11, 143–154.
- Jain, S.K., Singh, R., Sahu, B., 1997. Development of a liposome-based contraceptive system for intravaginal administration of progesterone. *Drug Develop. Ind. Pharm.* 23, 827–830.

- Kikuchi, H., Yamauchi, H., Hirota, S., 1994. A polyol dilution method for mass production of liposomes. *J. Liposome Res.* 4, 71–91.
- Knuth, K., Amiji, M., Robinson, J., 1993. Hydrogel delivery systems for vaginal and oral applications. *Adv. Drug Deliv. Rev.* 11, 137–167.
- Kükner, S., Ergin, T., Cicek, N., Ugur, M., Yesilyurt, H., Gökmen, O., 1996. Treatment of vaginitis. *Int. J. Gynecol. Obstet.* 52, 43–47.
- Livengood, C.H., McGregor, J.A., Soper, D.E., Newton, E., Thomason, J.L., 1994. Bacterial vaginosis: efficacy and safety of intravaginal metronidazole treatment. *Am. J. Obstet. Gynaecol.* 170, 759–764.
- Mafhouz, N.M., Hassan, M.A., 2001. Synthesis, chemical and enzymatic hydrolysis, and bioavailability evaluation in rabbits of metronidazole amino acid ester prodrugs with enhanced water solubility. *J. Pharm. Pharmacol.* 53, 841–848.
- Owen, D.H., Katz, D.F., 1999. A vaginal fluid simulant. *Contraception* 59, 91–95.
- Pavelić, Ž., Škalko-Basnet, N., Schubert, R., Jalšenjak, I., 2004. Liposomal gels for vaginal drug delivery. In: Düzgünes, N. (Ed.), *Liposomes, Part D, in Methods in Enzymology*, 387. Elsevier Academic Press, San Diego, pp. 287–299.
- Pavelić, Ž., Škalko, N., Jalšenjak, I., 1998. Liposomes for vaginal drug delivery. *J. Liposome Res.* 8, 94–95.
- Pavelić, Ž., Škalko-Basnet, N., Jalšenjak, I., 1999. Liposomes containing drugs for treatment of vaginal infections. *Eur. J. Pharm. Sci.* 8, 345–351.
- Pavelić, Ž., Škalko-Basnet, N., Schubert, R., 2001. Liposomal gels for vaginal drug delivery. *Int. J. Pharm.* 219, 139–149.
- Perrett, S., Golding, M., Williams, W.P., 1991. A simple method for the preparation of liposomes for pharmaceutical applications: characterisation of the liposomes. *J. Pharm. Pharmacol.* 43, 154–161.
- Peschka, R., Dennehy, C., Szoka, F.C., 1998. A simple in vitro model to study the release kinetics of liposome encapsulated material. *J. Control. Release* 56, 41–51.
- Richardson, J.L., Whetstone, J., Fisher, A.N., Watts, P., Farraj, N.F., Hinchcliffe, M., Benedetti, L., Illum, L., 1996. Gamma-scintigraphy as a novel method to study distribution and retention of a bioadhesive vaginal delivery in sheep. *J. Control. Release* 42, 133–142.
- Škalko, N., Čajkovac, M., Jalšenjak, I., 1998. Liposomes with metronidazole for topical use: the choice of preparation method and vehicle. *J. Liposome Res.* 8, 283–293.
- Škalko-Basnet, N., Pavelić, Ž., Bećirević-Laćan, M., 2000. Liposomes containing drug and cyclodextrin prepared by the one-step spray-drying method. *Drug Dev. Ind. Pharm.* 26, 1279–1284.