Desarrollo de Sistemas Lipídicos Nanoestructurados de Halobetasol propionato para aplicación tópica

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Facultad de Farmacia y Ciencias de la Alimentación

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"Señor Fennyman, permítame que le explique el negocio del teatro. Por natural condición es una sucesión de obstáculos que conducen a un inminente desastre.

– ¿Qué haremos, pues?

– Nada… Extrañamente, siempre sale bien.

– ¿Cómo?

– No lo sé. Es un misterio.

Geoffrey Rush.

("Shakespeare in love", John Madden, 1998)

Cómo la vida misma.

AGRADECIMIENTOS

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ÍNDICE

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ABREVIATURAS

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AA	Ácido araquidónico
BS	Backscatering
Cb	Carbopol (Carbómero)
cLipid	Concentración de lípido
COX	Ciclooxigenasa
CYP	Citocromo
DLS	Dispersión dinámica de la luz
DoE	Diseño de experimentos
DSC	Calorimetría diferencial de barrido
EC	Estrato córneo
EE	Eficiencia de encapsulación
FTIR	Espectroscopia de infrarrojo con transformada de Fourier
HaCaT	Línea celular de queratinocitos humanos
HB	Halobetasol
HB-NLC	Nanocarrier lipídico cargado con Halobetasol propionato
LOX	Lipoxigenasa
LPS	Lipopolisacárido
LT	Leucotrieno
MW	Peso molecular
NLC	Nanocarrier lipídico
P1	Coeficiente de partición
P2	Coeficiente de difusión
PG	Prostaglandina
PI	Índice de polidispersión
Pl	Pluronic (Poloxamer)

ABREVIATURAS

FLA_2	Fosfolipasa A2
SLN	Nanopartículas lipídicas sólidas
TEM	Microscopia electrónica de trasmisión
THP-1	Línea celular monocítica humana
TNF	Factor de Necrosis Tumoral
TX	Tromboxano
XRD	Espectroscopia de difracción de rayos X
Zav	Tamaño promedio de partícula
ZP	Potencial zeta

RESUMEN - ABSTRACT

RESUMEN

Existe una amplia variedad de alternativas terapéuticas para el tratamiento de las enfermedades inflamatorias dérmicas que permiten una aproximación local o sistémica dependiendo de la severidad de los síntomas. El uso de corticoides tópicos es una de las alternativas más utilizada debido a su elevada eficacia y facilidad de dosificación, pero debido a la alta capacidad que poseen de atravesar los diferentes estratos de la piel, la gran problemática que presentan es la generación de efectos adversos sistémicos, dificultando un tratamiento seguro a largo plazo. El Halobetasol propionato (HB) es un corticoide de elevada potencia antiinflamatoria, utilizado en enfermedades crónicas como la psoriasis, a una concentración de 0.05% en geles, cremas y lociones, recomendándose su uso por un periodo no superior a 14 días consecutivos. Considerando lo anterior, el objetivo principal de esta investigación fue el desarrollo de un sistema nanoestructurado lipídico conteniendo Halobetasol propionato (HB-NLC) que promoviera la eficacia local antiinflamatoria reduciendo los efectos sistémicos. Las HB-NLC, fueron incorporadas en tres geles de aplicación dérmica que presentan alternativas de tratamiento dependiendo de las condiciones específicas de la piel del paciente. Las HB-NLC y los tres geles desarrollados, presentaron características fisicoquímicas adecuadas para la administración dérmica. Los perfiles biofarmacéuticos de HB-NLC y de los geles demostraron una liberación sostenida del fármaco y una penetración más lenta a través de la piel en comparación al fármaco libre. Las cuatro formulaciones desarrolladas, mostraron una óptima tolerancia dérmica, respaldada por los resultados *in vitro* de citotoxicidad en cultivos celulares HaCaT e *in vivo* de

test de Draize. Los ensayos de eficacia terapéutica *in vitro, ex vivo* e *in vivo*, demostraron que HB-NLC 0.01% presenta alta eficacia antiinflamatoria. Finalmente, los estudios de las propiedades biomecánicas, pusieron de relieve que los geles tienen acción hidratante y protectora sobre la piel. En conclusión y considerando los resultados obtenidos, el sistema lipídico nanoestructurado de HB y los geles desarrollados, constituirían una alternativa prometedora para el tratamiento local de enfermedades inflamatorias dérmicas, presentando estos últimos la ventaja adicional de mejorar las características de la piel, aumentando el confort de la zona afectada.

ABSTRACT

There is a wide variety of therapeutic alternatives for the treatment of dermal inflammatory diseases that allow a local or systemic approach depending on the symptoms severity. The topical corticosteroids are among the most widely used alternatives thanks to its high efficacy and ease of dosing, but due to their high capacity to cross the skin different layers, the great problem they present is the generation of systemic adverse effects after a few days of use, making safe long-term treatment difficult. Halobetasol propionate (HB) is a very high potency corticosteroid used in chronic diseases such as psoriasis, at a concentration of 0.05% in gels, creams and lotions, and its use is recommended for a period no longer than 14 consecutive days. Considering the above, the main aim of this research was the development of a lipid nanostructured system containing Halobetasol propionate (HB-NLC) that would promote local anti-inflammatory efficacy over the systemic action of the drug. HB-NLCs were incorporated into three gels for dermal application that present treatment alternatives depending on the specific conditions of the patient's skin. The HB-NLC and the three gels developed, presented adequate physicochemical characteristics for dermal administration. Biopharmaceutical profiles of HB-NLC and gels demonstrated sustained drug release and slower penetration through the skin compared to free drug. All four developed formulations presented optimal dermal tolerance, supported by *in vitro* cytotoxicity results in HaCaT cell cultures and *in vivo* Draize test. *The in vitro, ex vivo* and *in vivo* therapeutic efficacy trials demonstrated that HB-NLC 0.01% has high anti-inflammatory efficacy. Finally, studies of the biomechanical properties exhibited that the gels have a moisturizing and protective action on the skin.

In conclusion and considering the obtained results, the HB nanostructured lipid system and the gels developed would constitute a promising alternative for the local treatment of inflammatory dermal diseases, the latter, presenting the additional advantage of improving the characteristics of the skin, increasing the affected area comfort feeling.

1.

INTRODUCCIÓN

1.1. Anatomía y fisiología de la piel

La piel, que representa un 15% del peso corporal total, es el órgano de mayor tamaño del cuerpo humano y su principal función reside en dar soporte y mantener la homeóstasis de los órganos internos, y protegerlos del medio externo. De entre sus múltiples misiones destacan: recubrir la musculatura, huesos, ligamentos y órganos, mantener las condiciones internas estables, proteger de la pérdida de agua, de las abrasiones, de los cambios de temperatura y de agentes patógenos como bacterias, virus y sustancias químicas potencialmente nocivas (Proksch et al., 2008).

Desde el punto de vista químico, la piel está compuesta por un 70% de agua, 25% de proteínas, 2% de lípidos y 3% de otros compuestos. Anatómicamente, la piel se caracteriza por estar organizada en tres capas principales denominadas desde el exterior al interior: epidermis, dermis e hipodermis (Fore, 2006).

La **epidermis** corresponde a la capa más externa de la piel y su grosor varía entre 0.04 y 1.6 mm. Está compuesta principalmente por células queratinocíticas (más del 80%), pero contiene también otros tipos celulares como: melanocitos (encargados de la producción de melanina y de la protección natural contra los rayos UV), células de Merkel (células conectadas a terminaciones nerviosas de la piel responsables de las sensaciones táctiles) y células de Langerhans (célula epidérmica que posee marcadores de superficie característicos de las células inmuno-competentes).

En esta sección se alojan también los “anexos cutáneos” que comprenden a las glándulas sebáceas - responsables de la producción de sebo y mantenimiento del manto hidrolipídico de la piel-, glándulas sudoríparas -encargadas de eliminar substancias de desecho en forma de sudor y colaborar en la mantenimiento de la temperatura corporal-, los folículos pilosos - permiten el crecimiento del cabello y vellos corporales- y las uñas - caracterizadas por tener función sensitiva y protectora de las terminaciones nerviosas de los dedos, además de ser utilizadas como pinzas (James et al., 2011; Merad et al., 2008; Tanner y Marks, 2008).

La epidermis comprende 4 capas diferentes: estrato basal, estrato espinoso, estrato granuloso y estrato córneo.

El estrato basal está formado por una única capa de queratinocitos cuya función es la generación de nuevos queratinocitos mediante mitosis. Corresponde a la capa más interna de la epidermis y se encuentra junto a la dermis.

El estrato espinoso está formado por entre 3 y 10 capas de queratinocitos que emigraron desde el estrato inferior basal y su función principal es la producción activa de queratina.

El estrato granuloso está formado por queratinocitos que poseen gránulos de queratina en el citoplasma y que han perdido el núcleo, por lo que es considerado la primera capa de la epidermis formada por células muertas. En las zonas del cuerpo como palma de las manos y planta de los pies, esta capa puede ser más gruesa y denominarse estrato lúcido (Hendriks y Hendriks, 2001; Fitzpatrick y Morelli, 2010).

Finalmente, el estrato córneo (EC) está compuesto principalmente por corneocitos, que corresponden a queratinocitos diferenciados biológicamente muertos, unidos entre sí por corneodesmosomas. Es una capa hidrofóbica y comprimida de corneocitos, cuya principal función es proteger de la pérdida de agua y de la entrada de sustancias externas. Esta función, queda determinada principalmente por la constante descamación y renovación del EC, que en una epidermis saludable sucede cada 28 días (Fore, 2006; Tanner y Marks, 2008).

La **dermis** tiene un grosor de entre 0.3 y 3 mm, que varía dependiendo de la zona del cuerpo en la que se encuentre. Es una capa hidrofóbica compuesta en un 90% por colágeno - responsable del mantenimiento estructural y soporte de la piel-, fibras de elastina - que confieren la resistencia mecánica de la piel-, vasos linfáticos, glándulas sudoríparas y sebáceas. El 10% restante, corresponde a células tales como fibrocitos, monocitos, linfocitos, histiocitos y otras células sanguíneas (Jepps et al., 2013).

La **hipodermis** es la capa más profunda de la piel, puede representar entre el 15 y el 30% del peso corporal y tiene por principal función el aislamiento térmico, dar movilidad a la piel y actuar de reserva nutricional. Está compuesta por adipocitos y tejido conectivo, que provee a la piel con nervios y vasos sanguíneos. Su grosor puede variar entre diferentes partes del cuerpo y entre cada individuo (Ng y Lau, 2015).

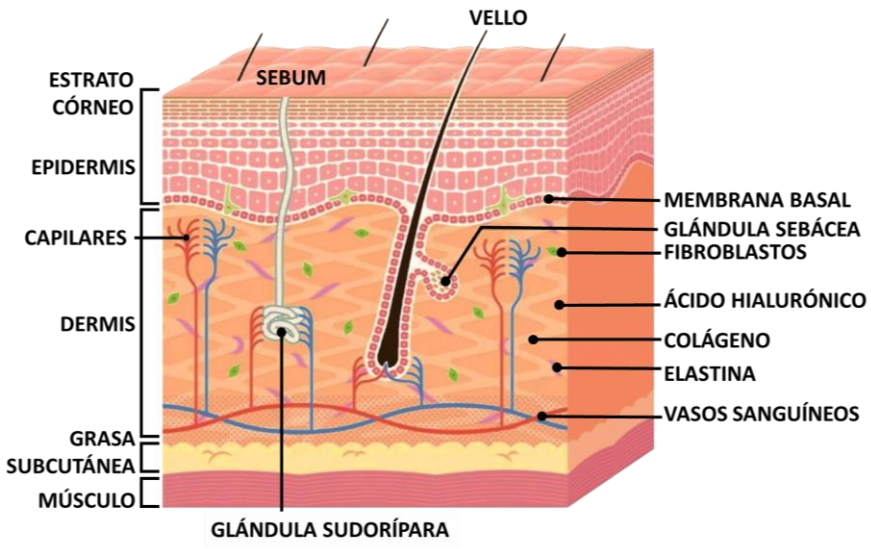

Figura 1. Estructura de la piel humana.

1.2. Penetración transdérmica

Un fármaco administrado por vía dérmica para el tratamiento local de una afección en la piel, se enfrenta a diversos procesos anatómicos, fisiológicos, bioquímicos y farmacocinéticos entre otros, que podrían dificultar el cumplimiento de su acción (Doménech et al., 2013).

La permeación transdérmica conlleva el movimiento de un fármaco desde el medio externo hasta el medio interno. Este proceso, implica el paso a través de las diferentes capas de la piel y sucede principalmente en tres etapas:

1. **Penetración**: proceso mediante el cual el fármaco entra al EC. En esta etapa, el vehículo debe tener una primera interacción con la piel para generar una interfase entre ambos que permita mediante difusión el acercamiento de la sustancia a la barrera (Mbah et al., 2011; Ng y Lau, 2015).

2. **Permeación**: mediante difusión pasiva, la sustancia cruza las capas de la piel, desde un compartimiento de mayor concentración a otro de menor concentración (desde la zona más externa a la más interna). Este proceso puede suceder de dos maneras diferentes:

    - *Vía transepidermal*: corresponde a la vía más importante, debido a que se produce directamente por la interacción de la sustancia con las células del EC. Este mecanismo de permeación se muestra dependiente de las características del fármaco, como lipofilia, pH, coeficiente de partición o pKa y de las condiciones del estrato córneo como nivel de hidratación, grosor y buen/mal estado entre otros (Barry, 2001). En este caso, el paso del fármaco puede ser por vía intracelular, preferente para moléculas hidrófilas que son capaces de atravesar las membranas celulares. O por vía intercelular, preferente para moléculas hidrófobas debido a que su paso sucede entre las matrices lipídicas celulares (Bolzinger et al., 2012).

- *Vía apendicular*: aunque más rápida, se considera una vía minoritaria debido a que la superficie corporal posee menos de un 1% de apéndices que generan una alteración en la continuidad del EC. La entrada de las sustancias se ve favorecida a través de los canales de las glándulas sudoríparas, glándulas sebáceas y folículos pilosos. Es una alternativa de acceso importante para moléculas de gran peso molecular, sustancias hidrófilas y drogas esteroidales (Tanner y Marks, 2008; Bolzinger et al., 2012; Williams y Barry, 2012).

3. **Absorción**: es el paso del fármaco a los vasos sanguíneos y al sistema linfático. Generalmente alcanzada esta etapa, se considera que la sustancia ha llegado a circulación sistémica y por tanto es capaz de ejercer más que un efecto localizado (Mbah et al., 2011).

Este proceso de penetración/permeación/absorción, se puede ver influenciado tanto por factores intrínsecos de la piel, como por las características intrínsecas del fármaco:

Factores relacionados con la piel:

- *Hidratación y edad*: Con la edad, la hidratación de la piel y la actividad enzimática local disminuye. Cuando el EC se encuentra deshidratado, se produce una descamación de las células superficiales y la absorción se ve alterada, mientras que si el EC se encuentra hidratado, aumenta la permeabilidad de los

fármacos, sobre todo si existe efecto oclusivo. En pieles de edades más avanzadas existe también una disminución del flujo sanguíneo, lo que provoca que el fármaco no pueda difundir libremente debido a que la sustancia se mantiene más tiempo en la dermis limitando su absorción y paso a circulación sistémica (Behl et al., 1980; Bronaugh y Maibach, 1999).

- *pH y zona corporal*: el valor de pH considerado eudérmico oscila de forma general entre los 4.0 – 6.5. Puede variar en un mismo individuo dependiendo de la zona corporal y puede verse afectado por factores internos como la edad, la genética, las hormonas e incluso por factores externos como temperatura, productos cosméticos y la contaminación ambiental. La leve acidez que presenta naturalmente la piel, puede afectar directamente el grado de disociación de un fármaco y por tanto, interferir en su habilidad para atravesar las membranas. Del mismo modo que resulta más difícil la penetración en zonas más gruesas como la planta del pie, la absorción se ve favorecida en zonas donde la piel es más delgada, como en la zona de la cara y la cabeza (Wilhelm et al., 1991; Bronaugh y Maibach, 1999).

- *Patologías*: las patologías dérmicas pueden afectar la absorción de sustancias disminuyéndola o generalmente aumentándola, debido a que si se genera una lesión física, las uniones moleculares se ven alteradas y aumenta el espacio intercelular, lo que provoca una alteración de la integridad y de la continuidad

del EC, permitiendo la entrada de sustancias xenobióticas, fármacos o microorganismos. Algo similar ocurre con las agresiones químicas y la inflamación (Hsieh, 1993).

- *Temperatura*: un aumento de 3 °C de la temperatura corporal aumenta el flujo sanguíneo y genera alteraciones en la conformación de los lípidos del EC hacia un empaquetamiento más laxo promoviendo el movimiento de las moléculas y provocando aumento de la permeabilidad a las sustancias (Bolzinger et al., 2012).

Factores relacionados con la estructura del fármaco y del vehículo.

- *Concentración del fármaco*: la concentración inicial del fármaco influye directamente en la creación de un gradiente de concentración para favorecer el proceso de difusión pasiva para penetrar y permear la piel (Naik et al., 2000).

- *Coeficiente de reparto*: se refiere a la afinidad que tiene el fármaco por la fase polar o apolar de dos líquidos inmiscibles. Para su cálculo experimental se utiliza una ecuación predictiva del coeficiente basado en la utilización de octano como solvente hidrófobo y agua como solvente hidrófilo. El resultado se expresa como logP y es significativo para determinar la interacción entre el fármaco y el EC que actúa como una membrana lipídica. Se considera que con valores de logP entre

1-3, un fármaco sería un buen candidato para permeación a través de la piel (Potts y Guy, 1992).

- *Coeficiente de permeabilidad*: es un parámetro proporcional al coeficiente de reparto del fármaco e inversamente proporcional al tamaño. Se refiere a la velocidad con que un fármaco difunde en un medio determinado, en este caso el EC (Moser et al., 2001).

- *Excipientes*: la matriz de la formulación puede modificar la permeación del fármaco, alterando por ejemplo su ionización. Referente al EC, el vehículo puede afectar la estructura aumentando su hidratación, alterando la organización lipídica o aumentando la temperatura, provocando una alteración en la función de barrera del EC y por tanto aumentando la absorción.

- *Propiedades reológicas de la formulación*: factores como la viscosidad o el efecto oclusivo de una formulación muy oleosa, pueden modificar la difusión del fármaco. A menor viscosidad, menor resistencia y por tanto, mayor difusión del fármaco desde la formulación al EC. La oclusión aumenta la hidratación del EC, comprometiendo la función de barrera y pudiendo aumentar la absorción a través de la piel hasta un 50% en comparación al mismo compuesto aplicado sin oclusión. (Zhai y Maibach, 2002).

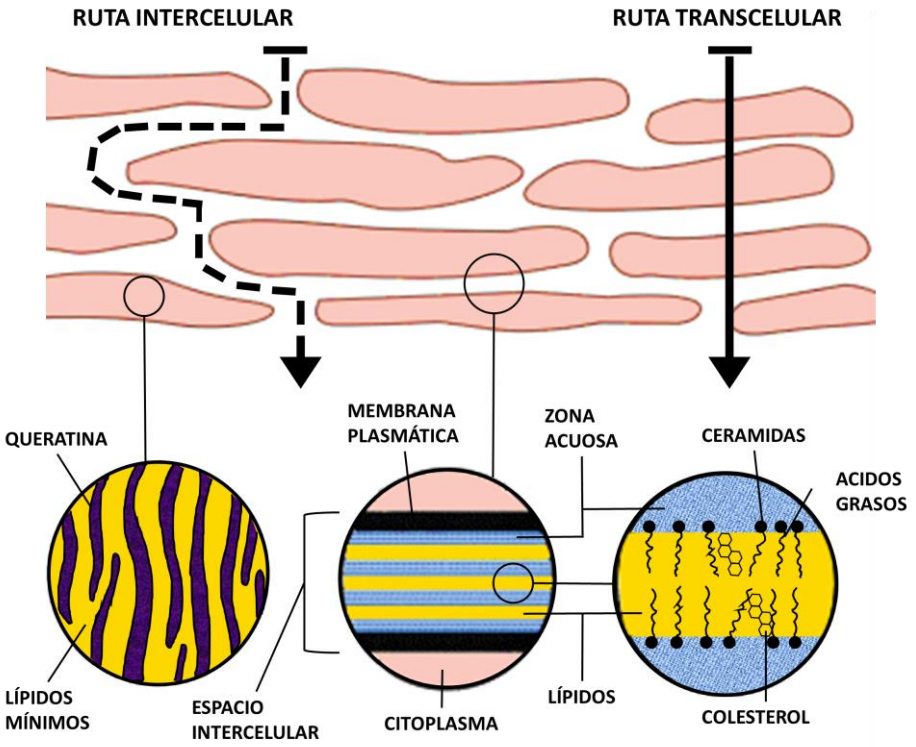

Figura 2. Vías de penetración de sustancias a través de la piel. (Adaptado de Barry, 2001).

1.3. Moduladores de la permeabilidad: Promotores de penetración

Corresponden a sustancias farmacológicamente inertes que promueven la absorción de los fármacos administrados por vía dérmica. Son comúnmente utilizados de forma individual o en mezclas para alterar la función barrera de la piel y permitir que la absorción del fármaco a partir de esta vía sea más efectiva. Para considerarse adecuada, la

molécula “aceleradora de la penetración” debe cumplir una serie de requisitos tales como:

- *Compatibilidad química*: la molécula debe ser químicamente compatible con el fármaco y con todos los componentes de la formulación, lo que significa que no debe alterar la estructura ni el funcionamiento natural del fármaco y no debe interaccionar de forma alguna con los excipientes.

- *Seguridad*: la molécula no debe ser tóxica, ni irritante ni alérgeno por contacto directo con la piel. Durante su uso, no debe provocar la pérdida de líquidos corporales, electrolitos ni ningún otro material endógeno. Finalmente, para su selección y utilización, debe asegurarse previamente su calidad y pureza para uso humano.

- *Función*: su efecto promotor de la penetración debe ser predecible, efectivo únicamente a corto plazo y siempre reversible, lo que significa que al dejar de estar en contacto con la piel, ésta debe volver a sus características y funcionalidad de barrera originales (Kanikkannan et al., 2000).

Los mecanismos mediante los cuales estas moléculas alteran la función de barrera del EC son variados y dependen principalmente de sus características fisicoquímicas, y de las características intrínsecas del fármaco. De forma general, el mecanismo está basado en aumentar la fluidez del EC (Singh y Singh, 2000).

Tabla 1. Clasificación química y mecanismo de acción de los promotores de penetración más utilizados.

CLASIFICACIÓN QUÍMICA	MECANISMO DE ACCIÓN PROMOTOR
Ácido graso	Provocar disrupción del empaquetado lipídico disminuyendo la resistencia a la difusión
Terpenos	Mejorar el coeficiente de partición vehículo/ EC del fármaco
Lactamas	Intercalar entre los lípidos alterando la estructura continua de la membrana
Urea	Inducir ruptura de la queratina generando poros hidrófilos en la membrana
Tensoactivos	Alterar la estructura de lípidos y proteínas desorganizando la membrana
Alcohol	Extraer los componentes lipídicos y proteínas generando poros en la membrana
Sulfóxidos	Alterar la conformación de la α-queratina e interaccionar con los lípidos aumentando la fluidez de la membrana
Pirrolidonas	Desnaturalizar la α-queratina, reteniendo agua e interaccionando con los lípidos aumentando la fluidez de la membrana del EC.

1.4. Respuesta inflamatoria

El proceso inflamatorio es una respuesta inmunológica genérica e inespecífica del organismo frente a la presencia de agentes patógenos, sustancias xenobióticas, tóxicas, irritantes, como resultado de un trauma o de una enfermedad sistémica inflamatoria autoinmune. Esta reacción puede variar en su intensidad y duración y viene generalmente acompañada de calor, sudoración, enrojecimiento, dolor y perdida de la función local. Su finalidad es diluir, eliminar o aislar el agente etiológico que origina la alteración y reparar el daño a nivel local (Dennis y Norris, 2015; Gallo et al., 2017).

A partir de los lípidos de membrana y por efecto de la Fosfolipasa A2 (FLA2), se libera el ácido araquidónico (AA), precursor de los eicosanoides y mediador celular de la respuesta inflamatoria que puede estimular la producción de citoquinas pro-inflamatorias y los procesos apoptóticos. El AA puede metabolizarse mediante la vía de la *ciclooxigenasa (COX)* generando prostaglandinas (PGs) y tromboxanos (TXs); por la vía de la lipooxigenasa (LOX) produciendo leucotrienos (LTs) y otros metabolitos intermediarios y finalmente por la vía del citrocromo P450 (CYP450), dando origen al ácido epoxieicosatetraenoico (EETs).

Los eicosanoides producidos a partir del AA cumplen importantes funciones homeostáticas en los procesos inflamatorios e inmunopatológicos que van desde la modulación de la fuga vascular hasta la regulación de la agregación plaquetaria (White, 1999; Dennis y Norris, 2015).

Tabla 2. Respuestas fisiológicas en la inflamación.

VÍA METABÓLICA	MEDIADOR	EFECTO BIOQUÍMICO Y FISIOLÓGICO
COX	*PGD_2*	Vasodilatación; maduración de mastocitos. ↑Reclutamiento de eosinófilos y respuesta alérgica.
	PGE_2	Vasodilatación y permeabilidad vascular (enrojecimiento y ↑T a nivel dérmico). Hiperalgesia; ↑IL-10; ↓TNF.
	PGI_2	↓Agregación plaquetaria; hiperalgesia; vasodilatación; ↑IL-10; ↓TNF.
	TXA_2	↑Agregación plaquetaria; vasoconstricción.
5-LOX	*LTB_4*	Reclutamiento de neutrófilos; permeabilidad vascular. Mejora de la función de barrera epitelial.
	LTC_4, LTD_4 y LTE_4	Permeabilidad vascular; Extravasación de neutrófilos.
	HPETEs, HETEs y diHETEs	Hiperalgesia.
	LXA_4, 15-epi-LXA_4, LXB_4 y15-epi-LXB_4	Reclutamiento de neutrófilos; esferocitosis.
CYP	*EETs*	Vasodilatación; antihiperalgesia; ↓expresión COX-2.

La inflamación, es una respuesta fisiológica que puede considerarse una extensión de la capacidad auto-reguladora del organismo ayudando a mantener y reparar el estado funcional saludable del tejido. Cuando este proceso no es capaz de limitarse a una duración-zona determinada, deja de considerarse una respuesta aguda y puede evolucionar de forma crónica. La inflamación crónica, forma parte en patologías de la piel tales como dermatitis atópica, eccema, vitíligo y psoriasis. En todas estas alteraciones, generalmente coexisten la respuesta inflamatoria crónica y la respuesta inmune alterada, lo que finalmente conlleva a una modificación constante en el EC que produce una alteración en su función de barrera, aumentando en exceso la pérdida de agua y de hidratación natural de la piel y disminuyendo su capacidad de protección frente a agentes externos (Leung et al., 2004; Lawton, 2009; Reich, 2012).

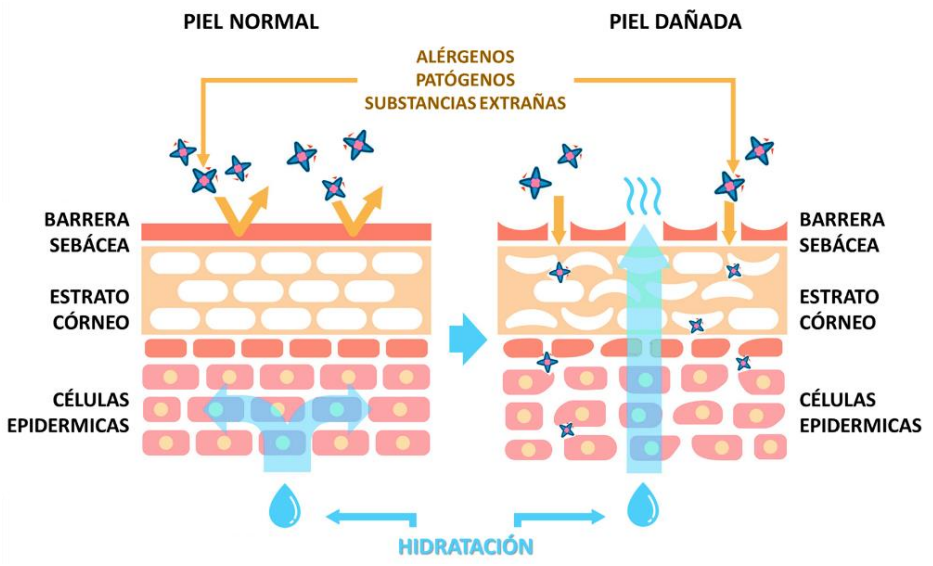

Figura 3. Diagrama de barrera cutánea alterada.

1.5. Antiinflamatorios esteroideos de aplicación tópica: Halobetasol

Los antiinflamatorios esteroideos corresponden a sustancias sintetizadas artificialmente cuya estructura química base, el ciclopentanoperhidrofenantreno, ha sido modificada mediante procesos de halogenización y esterificación para modular sus efectos antiinflamatorios, inmunosupresores, mineralo-corticoideos y anti-proliferativos. Existe una amplia variedad de esteroides tópicos disponibles que difieren en su potencia y formulación, se encuentran entre los fármacos más comúnmente utilizados y son considerados de los más eficaces en el tratamiento de enfermedades dérmicas como psoriasis, dermatitis atópica, eccema y vitíligo (Grau, 2006; Ference y Last, 2009).

Los corticoides ejercen su acción al formar complejos con los receptores citoplasmáticos específicos de corticoides, que penetran en el núcleo celular y uniéndose al ADN, inhiben la transcripción de proteínas inflamatorias e inducen la expresión de proteínas anti-inflamatorias. Al activarse el receptor de corticoides se induce la Anexina I, proteína que provoca una inhibición de la acción de FLA2, impidiendo que el AA de las membranas celulares quede libre para ser metabolizado por COX y LOX, evitando la producción de eicosanoides pro-inflamatorios (PGs, TXs, LTs, entre otros). Paralelamente, se produce una inducción de la MAPK fosfatasa I que inhibe toda la cascada de MAPK por fosforilación de las proteínas, provocando inhibición indirecta de la FLA2. Este mecanismo anti-inflamatorio está acompañado por un bloqueo de los factores de transcripción pro-inflamatorios Jun y Fos y por una inhibición

de la actividad del factor de transcripción NF-kB, lo que produce una inhibición en la expresión de enzimas COX y otras citoquinas pro-inflamatorias (Rhen y Cidlowski, 2005; Barnes, 2006).

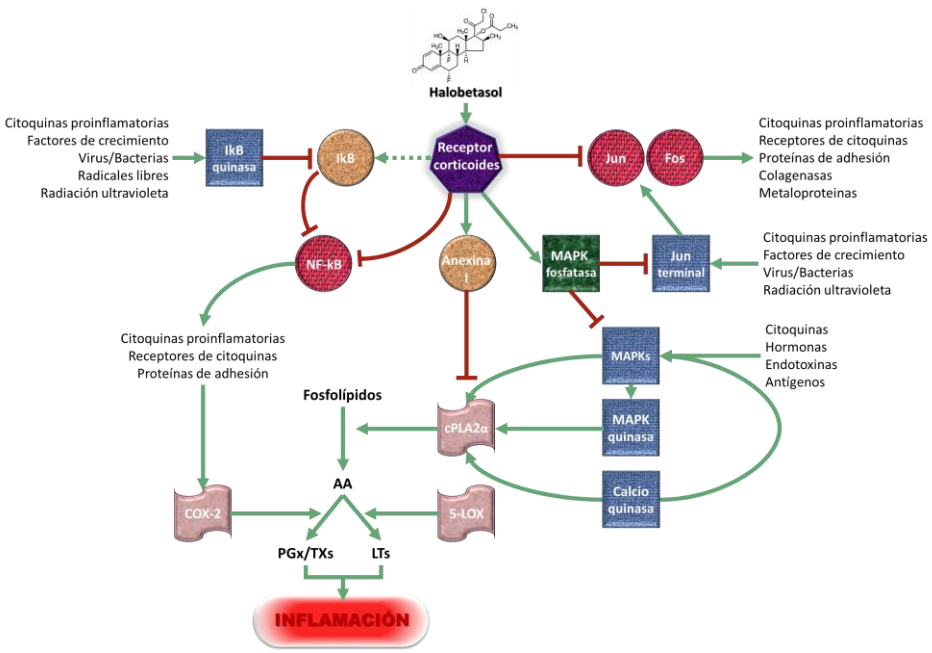

Figura 4. Mecanismo de acción de los corticoides.
(Adaptada desde Rhen y Cidlowski, 2005).

Si bien los corticoides son considerados potentes y efectivos anti-inflamatorios, producto de la amplia gama de mecanismos de transcripción que desencadenan pueden generar una extensión de efectos adversos de complejidad variable. Cuando son aplicados por vía tópica, a nivel local, y principalmente producto de su efecto mineralocorticoide y antiproliferativo sobre queratinocitos y fibroblastos, pueden producir (des)pigmentación; atrofia del tejido subcutáneo; disminución de la microvasculación; telangiectasias y foliculitis entre otras alteraciones

generalmente reversibles. Posterior a un uso dérmico prolongado y debido a la alta permeabilidad cutánea que presentan los corticoides a causa de su estructura y solubilidad en el SC, pueden generar también efectos no deseados a nivel sistémico. Dentro de los efectos adversos más reportados se encuentran: hiperglucemia, glaucoma e insuficiencia adrenal, alteraciones sistémicas y metabólicas que de no ser controladas a tiempo, a largo plazo pueden desencadenar en enfermedades crónicas incapacitantes (Kolbe et al., 2001; Serrano, 2006; Dey, 2014).

El Halobetasol propionato (HB) de fórmula $C_{25}H_{31}ClF_2O_5$, es un corticoide de uso externo considerado súper potente y utilizado para afecciones dérmicas (Hengge et al. 2006). Es una molécula de 485 g/mol con carácter lipófilo (LogP 3.82), prácticamente insoluble en agua (0.00757 mg/ml), con un punto de fusión entre 213-215 °C y constante de disociación de pKa 13.55 (National Center for Biotechnology Information, 2019). Las formas farmacéuticas actualmente comercializadas incluyen cremas, ungüentos y lociones, todas en una concentración del 0,05%. En todas las patologías en las cuales se indica este fármaco, (principalmente psoriasis), no se recomienda su uso por más de dos semanas consecutivas ni en zonas extensas de la piel, y se ha descrito categóricamente que debido a su alta potencia, por tratarse de un compuesto fluorado, no debería utilizarse en mucosas ni bajo oclusión. (Goldberg et al., 1991; Herz et al,. 1991; Emer et al., 2011; Prakash et al., 2015).

1.6. Sistemas lipídicos nanoestructurados de uso local: NLC

Las nanopartículas lipídicas, de tamaño comprendido entre 10 – 1.000 nm, representan un sistema transportador alternativo a los sistemas coloidales tradicionales, tales como emulsiones, liposomas, micro y nanopartículas poliméricas, manteniendo sus ventajas pero evitando algunas de sus principales problemáticas (Müller et al., 2000; (Ulbrich y Lamprecht, 2010).

Una de las principales ventajas que presentan estos transportadores lipídicos es su matriz sólida a temperatura ambiente, que tiene la capacidad de encapsular y estabilizar principios activos químicamente lábiles al agua u oxígeno, previniendo el proceso de degradación, alargando su vida útil y mejorando su biodisponibilidad, además, presenta una alta capacidad de carga de fármacos poco solubles en agua. Están conformadas por lípidos biocompatibles como triglicéridos y ácidos grasos, disminuyendo considerablemente los problemas de toxicidad que presentan las nanopartículas poliméricas (Shah et al., 2015).

Dentro de los activos que se han incorporado en partículas lipídicas encontramos fármacos y activos cosméticos tales como Timolol, Doxorrubicina, Diazepam, Deoxycorticosterona, ciclosporina, Aciclovir, ácido ferúlico, isotretinoina, perfumes, retinoides y tocoferol entre otros (Müller et al., 2000; Souto y Müller, 2008). Se han demostrado también, una serie de características favorables para su uso en piel, tanto en la industria farmacéutica como cosmética. Al ser una

formulación con componentes hidrofóbicos, tienen la facultad de generar una película protectora y reparadora sobre la piel, es decir, una monocapa fina que recubre la zona de aplicación. Este recubrimiento, produce un efecto oclusivo que aumenta la hidratación de la piel debido a que disminuye la pérdida de agua transepidermal (TEWL) y que generalmente promueve la absorción de los fármacos o compuestos incorporados en la partícula. Este efecto oclusivo es inversamente proporcional al tamaño de partícula, lo que significa que a menor tamaño de partícula, mayor oclusión de la superficie debido a la disminución del espacio inter-partícula generado. Esta acción permite que las partículas se depositen sobre la piel dañada, ayudando a su reparación (Souto y Müller, 2008).

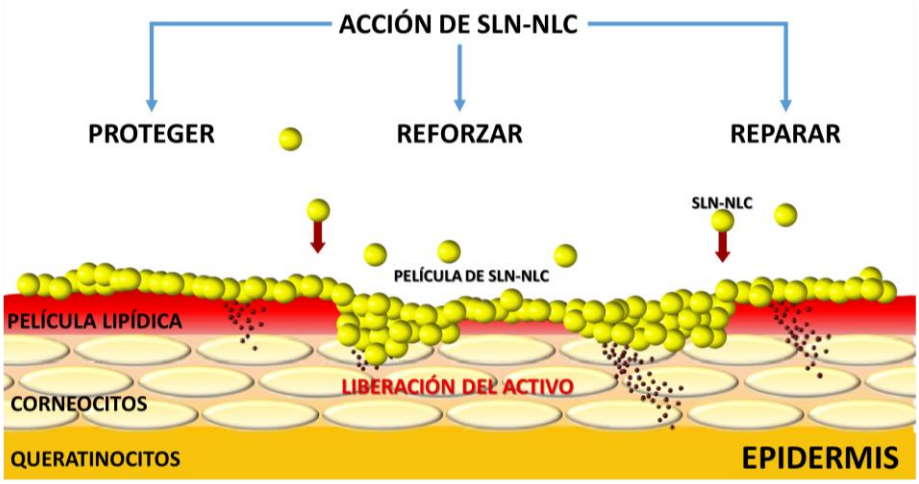

Figura 5. Propiedades de las nanopartículas lipídicas sobre la piel. (Adaptado de Müller et al., 2011).

Los sistemas lipídicos nanoestructurados se pueden dividir según su composición en dos generaciones: nanopartículas lipídicas sólidas (SLN) y nanotransportadores lipídicos (NLC).

Los SLN son partículas coloidales derivadas de emulsiones de aceite en agua que reemplazan los lípidos líquidos con una matriz de lípidos sólida a temperatura corporal y estabilizada mediante el uso de tensoactivos. Corresponden a la primera generación de nanopartículas lipídicas y están compuestas por uno o más lípidos sólidos mezclados entre sí. Si bien los SLN resultan efectivos como sistema de liberación controlada de fármacos, su principal problema radica en la cristalización (perfecta organización) de los lípidos a temperatura ambiente, porque limita el espacio disponible para la inclusión del fármaco y produce su rápida liberación durante la fase de almacenamiento de la formulación, por lo que el fármaco vuelve a quedar libre en el sistema acuoso. (Mehnert y Mäder, 2012).

Con la finalidad de solucionar esta problemática se desarrolló una segunda generación de nanopartículas lipídicas a partir de los SLN: los NLC. Corresponden a una mezcla de lípidos líquidos (aceites) y lípidos sólidos mezclados entre sí y dispersos en una solución acuosa que contiene uno o una mezcla de tensoactivos. La principal ventaja de los NLC sobre los SLN es que muchos fármacos son más solubles en un lípido líquido que en un lípido sólido (Severino et al., 2012).

La mezcla lipídica de los NLC se mantiene en estado sólido a temperatura ambiente pero con un punto de fusión inferior al de los lípidos sólidos que lo conforman. Al tratarse de una mezcla, la

cristalización lipídica sucede de forma más “desorganizada” generando espacios que permiten incorporar una mayor cantidad de fármaco en la nanopartícula, aumentando por tanto, la capacidad de carga y la estabilidad del fármaco durante el almacenamiento en comparación a los SLN (Müller, et al,. 2002; Czajkowska-Kośnik et al., 2019).

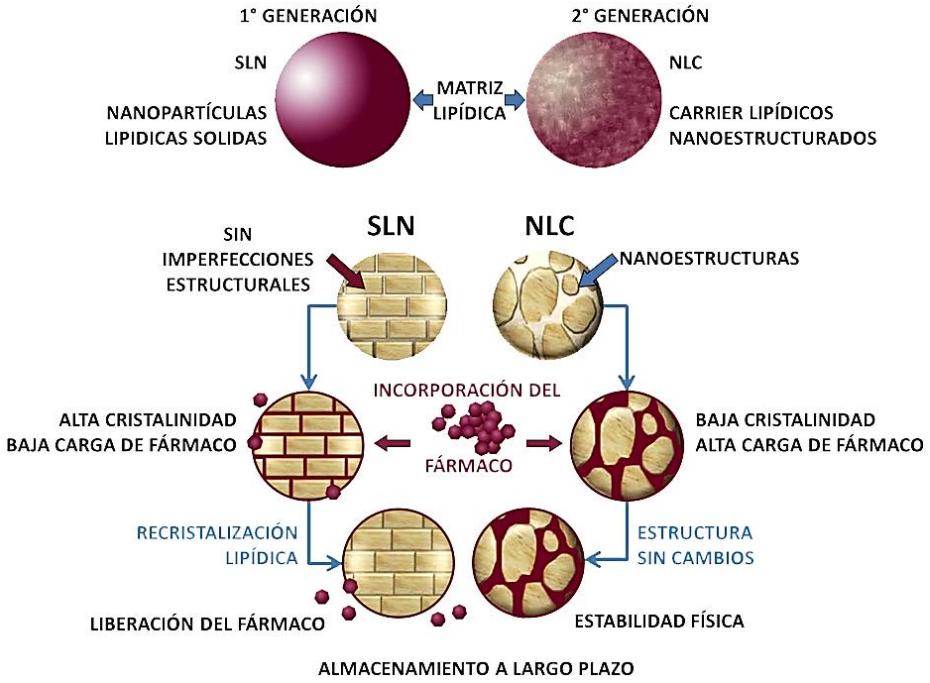

Figura 6. Diferencias estructurales y capacidad de carga de fármaco entre las matrices de SLN y NLC. (Adaptada desde Müller et al., 2011).

Los componentes utilizados para la fabricación de NLC son lípidos de características biodegradables, no tóxicos, bien tolerados y reconocidos como seguros para su uso humano por organismos internacionales (Kalepu et al., 2013; Sánchez-López et al., 2017; Czajkowska-Kośnik et al., 2019).

Tabla 3. Componentes lipídicos y tensoactivos utilizados en la producción de NLC para aplicación tópica.

Categoría	Excipiente
Componentes lipídicos estructurales	Triglicéridos (trilaurina, tricapriloína, tristearina, tripalmitina)
	Triglicéridos cápricos / caprílicos (Mygliol®), dicaprilocaprato de propilenglicol (Labrasol®)
	Diglicéridos (dipalmitina, distearina)
	Monoglicéridos (mono-estearato de glicerilo [Myvapex 600®], palmitostearato de glicerilo [Precirol® ATO])
	Alcoholes alifáticos (alcohol cetílico, alcohol estearílico)
	Ácidos grasos de cadenas C10-C12 (ácido decaenoico, ácido linoleico)
	Ésteres de polialcohol y colesterol (colesteril hemisuccinato, colesteril butirato y colesteril palmitato)
Componentes lipídicos tensoactivos	Polisorbatos: Tween 20® Tween 80®
	Poloxamer (188 – 407) [Lutrol 20®, Lutrol 80®]
	Polioxil 35 aceite de castor [Cremophor EL]
	Polioxietileno estearato [Mirj 52®]
	Sorbitan laurato , oleato [Span 20® – Span 80®]

La fabricación de NLC puede realizarse mediante varios métodos de baja y alta energía entre los que encontramos la micro-emulsión, la emulsión múltiple; la homogenización por ultrasonido; la precipitación por solvente y la homogenización a alta presión (Trotta et al., 2003; Date et al., 2011; Pucek et al., 2017).

La elección del método de fabricación debe hacerse considerando principalmente las características fisicoquímicas y estabilidad del fármaco que se desea encapsular, la finalidad de uso y características de las NLC deseadas, la estabilidad de los lípidos componentes y el volumen de producto final que se necesite.

El proceso de homogenización a alta presión permite trabajar a bajas o altas temperaturas según las características y resistencia de sus componentes. Presenta como principales ventajas la facilidad de escalado industrial, la no utilización de solventes orgánicos y la producción de dispersiones con partículas de pequeño diámetro e índice de polidispersión (PI) generalmente bajo de 0.2 en formulaciones de hasta un 40% de lípido (Severino et al., 2012).

Para producir NLC mediante esta técnica, el fármaco se disuelve en una mezcla fundida de un lípido sólido con un lípido líquido, calentados ligeramente por encima del punto de fusión de la mezcla. Posteriormente, la masa de lípidos que contiene el fármaco se dispersa en una solución de tensoactivo de idéntica temperatura mediante agitación a alta velocidad, produciendo una "pre-emulsión" acuosa de aceite en agua. Esta pre-emulsión se pasa a través de un homogeneizador de alta presión con temperatura controlada. Se ha descrito que un ciclo de homogenización a 500 bar sería suficiente para producir partículas de tamaño inferior a 300 nm, pero este paso, se puede repetir si se desea obtener partículas de menor tamaño o menor PI dependiendo de las características de la vía de administración. El posterior enfriamiento de

la nanoemulsión conduce a la cristalización del lípido y a la formación de los NLC en medio acuoso (Muchow et al., 2008).

La caracterización de NLC considera múltiples técnicas instrumentales para la determinación de los parámetros fisicoquímicos, biofarmacéuticos, de seguridad y eficacia, algunas de las cuales se describen a continuación (Hall et al., 2007; Lin et al., 2014).

Tabla 4. Principales técnicas para la evaluación y caracterización de las nanopartículas.

Método	Parámetro analizado
Caracterización fisicoquímica	
Espectroscopía de correlación fotónica (DLS)	Tamaño promedio y distribución de tamaño.
Microscopía electrónica de barrido (SEM) *Microscopía electrónica de transmisión (TEM)*	Forma, tamaño, distribución y agregación de las partículas.
Microscopía de fuerza atómica (AFM)	Tamaño y distribución, forma, estructura, agregación.
Espectroscopia infrarroja con transformada de Fourier (FTIR)	Conformación y estructura molecular, determinación de grupos funcionales
Difracción de rayos X (XRD)	Composición química de la superficie, cristalinidad.
Electroforesis	Carga superficial (potencial zeta).
Calorimetría de barrido diferencial (DSC)	Estado físico y posible interacción entre componentes.

2.

OBJETIVOS

OBJETIVOS

El principal objetivo de este trabajo se centra en el desarrollo y caracterización de un sistema lipídico nanostructurado (NLC), conteniendo Halobetasol para el tratamiento eficaz de enfermedades inflamatorias de la piel, evaluando el comportamiento biofarmacéutico, el perfil toxicológico y la eficacia terapéutica.

Objetivos Específicos

- Desarrollar y optimizar un sistema lipídico nanoestructurado conteniendo HB (NLC-HB) mediante un diseño factorial central compuesto.
- Determinar las características fisicoquímicas y la estabilidad del sistema HB-NLC optimizado.
- Estudiar el comportamiento biofarmacéutico del fármaco, en presencia de promotores e incorporado en el sistema lipídico.
- Evaluar la toxicidad y tolerancia del sistema HB-NLC mediante técnicas *in vitro* (cultivo celular) e *in vivo* (Test Draize).
- Evaluar la eficacia anti-inflamatoria *in vitro* (determinación de citoquinas), *in vivo* (estimulación con histamina) y *ex vivo* (RT-PCR) de HB-NLC.
- Incorporar HB-NLC a tres geles diferentes para aplicación dérmica.
- Determinar la eficacia y las propiedades biomecánicas de la piel bajo tratamiento con los geles dérmicos de HB-NLC.

3.

RESULTADOS

RESULTADOS

El desarrollo de la presente investigación, resultó en la generación de tres publicaciones en forma de artículos científicos.

3.1. Effect of Different Skin Penetration Promoters in Halobetasol Propionate Permeation and Retention in Human Skin.

3.2. Development of Halobetasol-loaded nanostructured lipid carrier for dermal administration: Optimization, physicochemical and biopharmaceutical behavior, and therapeutic efficacy.

3.3. Nanostructured lipid carriers loaded with Halobetasol propionate for topical treatment of inflammation: Development, characterization, biopharmaceutical behavior and therapeutic efficacy of gel dosage forms.

3.1. Effect of Different Skin Penetration Promoters in Halobetasol Propionate Permeation and Retention in Human Skin.

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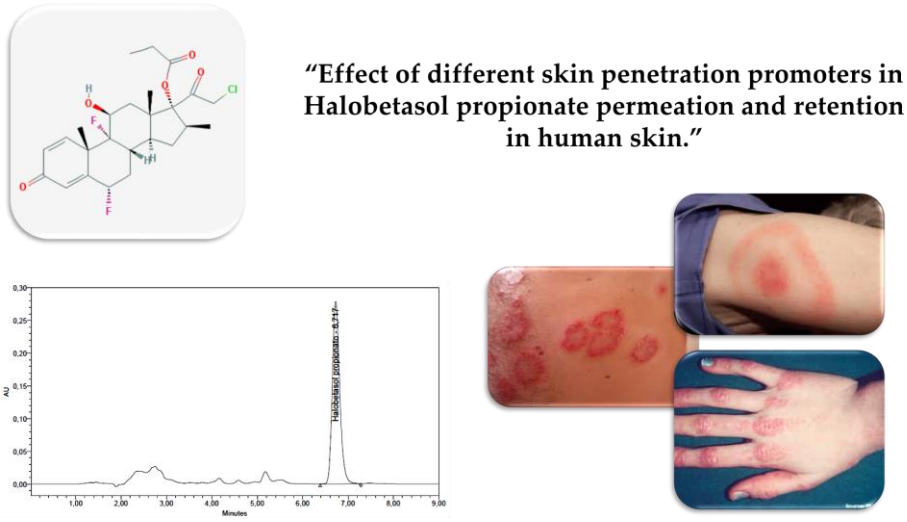
Resumen Gráfico



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Effect of Different Skin Penetration Promoters in Halobetasol Propionate Permeation and Retention in Human Skin

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Abstract: Halobetasol propionate (HB) is a potent synthetic corticosteroid used against inflammatory skin diseases, such as dermatitis, eczema, and psoriasis, among others. The aim of this study is to define how the presence of different skin penetration enhancers (nonane, menthone, limonene, azone, carene, decanol, linoleic acid and cetiol) affects the penetration and retention in skin of HB. To determine drug penetration through skin, 5% of each promoter was used in an ex vivo system with human skin on Franz cells. The results showed that the highest permeation occurs in the presence of menthone, followed by nonane. Permeation parameters were determined. The in vivo test was assessed, and the formulation containing HB-menthone presented better anti-inflammatory efficacy. These results are useful to generate a specific treatment according to each patient's needs, and the inflammatory characteristics of the disease.

Keywords: halobetasol propionate; permeation enhancers; skin permeation; skin inflammation; topical corticosteroid

1. Introduction

Skin is considered to be the largest organ of the human body, with a surface area of approximately 2 m^2. It has an integrated and complex composition that acts as a barrier against exogenous components [1]. This organ is divided into three main layers (starting with the deepest): hypodermis, dermis, and epidermis, the most superficial. The stratum corneum is to the outermost layer of epidermis, which is actually the main barrier to drug permeation [2–4]. Despite being a barrier, due to its properties and its structure being rich in capillaries and appendages, skin has been widely studied and used as a route for medication administration [5,6]. Skin can suffer varied alterations or diseases, such as dermatitis, eczema, and psoriasis, among others. A common feature of skin diseases is inflammation, redness, and edema. Although there are various alternative treatments for these symptoms, one of the drug groups of choice is that which is made up of those known as corticosteroids [7–10].

Corticosteroids are a widely used alternative treatment for inflammatory skin diseases. The drugs in this group can be classified according to their potency or route of administration. The most commonly used topical corticosteroids belong to class I, "superpower corticosteroids". Among them we find clobetasol, betamethasone, and halobetasol propionate (HB) [11]. Several clinical studies in humans have demonstrated the anti-inflammatory effectiveness of topical corticosteroids against

different skin diseases [12–15]. However, one of the great problems in treatment with topical corticosteroids is that due to their structure and lipophilicity, they can penetrate healthy skin and reach systemic circulation, and may cause toxicity and side effects such as Cushing syndrome, stretch marks, pruritus, acne, hypertension, glaucoma, glycosuria, and growth retardation. Moreover, permeation of corticosteroids may be greater in diseased or altered skin [11,16–20]. In general terms, drugs can permeate through the skin by three routes: the transcellular route, the intracellular route and the trans-appendix route [21]. The route used, and the amount of drug permeated through the skin, depends on the characteristics of the drug and its concentration, the condition of the skin, and external factors, such as occlusion or humidity [19]. In normal conditions, skin can avoid the entrance of external substances or let them pass passively (this is the case with corticosteroids). Due to this, to improve the effect of certain drugs and diminish side effects, it is necessary to modulate their permeability. For this purpose, several alternatives have been described, among which there are the permeation enhancers that can be used in a formulation for improving the transdermal drug delivery by reversibly decreasing the barrier resistance, and at the same time, some of them can be used for enhancing the drug retention in skin [22–25].

It has been described that these permeation enhancers must have certain properties: they must be well tolerated; they must rapidly begin their action, and they must be suitable for the formulation, the excipients, and for the active ingredient. Enhancers must not cause irreversible damage to the skin and must not have any pharmacological activity [24,26,27]. These enhancers can be classified into various groups depending on their mechanism of action, their structure, and their chemical characteristics. At a general level, some permeation enhancers and their chemical classification are presented in Table 1.

Table 1. Permeation enhancers and their chemical classification.

Chemical Class	Example(s)
Fatty acids	Oleic acid, Undecanoic acid, Linoleic acid
Alcohols and Alkanols	Octanol, Nonanol, Decanol
Terpenes	Menthol, Thymol, Limonene, Carene
Sulfoxides	Dimethyl sulfoxide, Dodecyl methyl sulfoxide
Surfactants	Sodium lauryl sulfate, Cetiol, Sorbitan mono-oleate
Polyols	Propylene glycol, Polyethylene glycol
Amides	*n*,*n*-Dimethyl-*m*-toluamide
Ureas	Urea
Lactam	Laurocapram (Azone®)
Sugars	Cyclodextrins

Modified from [28,29].

Among the possible mechanisms of action of the permeation enhancers, the following have been described: reversible disruption of the lipid matrix of the skin or on the domains of keratin, alteration in the partitioning coefficient of the drug with the tissue, and alteration of the skin metabolism [24,28]. In both the cosmetic and pharmaceutical industries, the promoters have been widely used and studied to modulate the permeation of various compounds, among them, lactam, terpenes, and alkanols [26,30,31]. Due to the differences between promoter groups, it is necessary to carry out a study to determine which would be the best performing promoter to modulate the penetration through skin, as this depends on the drug. All of the foregoing means that the aim of this study is to define how the presence of different skin penetration enhancers (nonane, menthone, limonene, azone, carene, decanol, linoleic acid, and cetiol) affects the HB penetration and retention in human skin in an ex vivo experiment. Working towards this purpose, we have developed eight different formulations containing the drug and the promoter. We developed and validated a simple, easy-to-use and reliable method for the quantification of HB in ex vivo experimentation using HPLC methodology. Finally, the therapeutic efficacy of the selected formulations was determined in vivo.

2. Results

2.1. HPLC Validation Methodology

The method was validated for specificity, accuracy, precision, linearity, and sensitivity to analyze HB from samples obtained in ex vivo experimentation on human skin.

Under the evaluated conditions described in the methodology, the method is considered specific for the detection and quantification of HB. Figure 1 shows that the chromatograms of the blanks used do not interfere with HB peak. The mean retention time of the drug was 6.6 min.

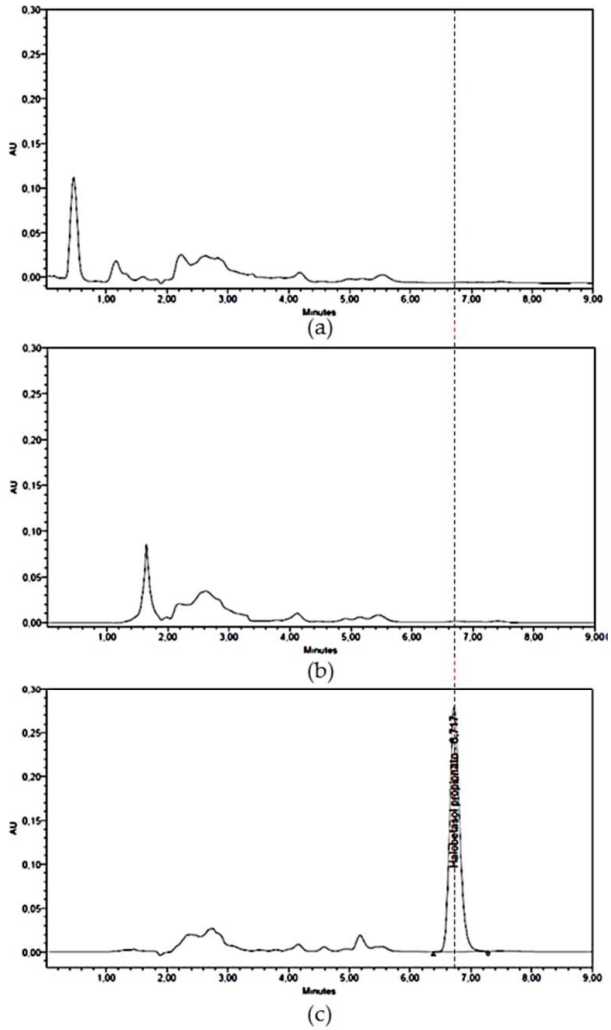
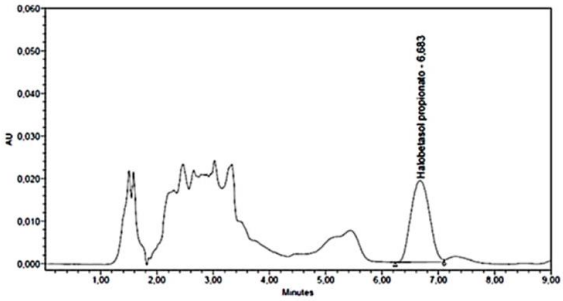


Figure 1. *Cont.*



(d)

Figure 1. Chromatograms of (**a**) blank of T/w; (**b**) permeated T/w; (**c**) standard 25 µg/mL halobetasol propionate (HB); (**d**) HB permeated in presence of enhancer menthone. (T/w: Transcutol®/water).

Accuracy and precision were assessed between the concentrations of 0.5–25 µg/mL. Acquired results are shown in Table 2, expressed as a percentage of relative error (RE) and coefficient of variation (CV). The obtained values for %RE and %CV did not go above 8% and 10% respectively, indicating that the analytical method is accurate and precise in the concentration range under study.

Table 2. Accuracy and precision inter-day data for HB standards solutions.

Standard Concentration (µg/mL)	Calculated Concentration (µg/mL)	%RE	%CV
0.50	0.46 ± 0.04	7.52	8.12
1.00	1.01 ± 0.09	−1.29	9.18
5.00	4.97 ± 0.06	0.55	1.11
10.00	10.00 ± 0.12	0.01	1.15
15.00	14.97 ± 0.16	0.18	1.06
20.00	20.15 ± 0.50	−0.73	2.48
25.00	24.90 ± 0.26	0.38	1.05

Linearity was established by the measurement of five calibration curves, and ranged between 0.5 and 25 µg/mL. Table 3 shows the areas obtained for each standard concentration of each curve. Statistical analysis ANOVA shows that there are no significant differences between the areas ($p = 0.6375$), indicating that the method is linear in the studied range. From these data, the linear equation is defined by $y = 116827x + 6030$, with $r^2 = 0.999$.

Table 3. HB standard curve and respective area response factor.

Concentration (µg/mL)	Ratio 1	Ratio 2	Ratio 3	Ratio 4	Ratio 5
0.5	120,731.26	102,721.10	121,211.86	146,101.52	121,400.90
1	127,756.00	107,598.30	118,281.10	124,086.20	149,836.40
5	117,370.32	111,500.02	115,936.50	124,473.88	118,584.80
10	116,073.00	111,987.70	116,643.40	123,272.40	119,428.40
15	115,639.87	112,150.33	115,545.20	122,871.87	119,042.87
20	120,423.60	117,231.60	115,496.90	117,671.60	118,850.15
25	115,293.64	112,280.36	114,267.60	122,551.44	118,734.48

The results of the limit of detection (LOD) and the quantification limit (LOQ) obtained from the lineal equation are 0.34 ± 0.14 and 1.04 ± 0.44, respectively. Considering the results obtained for the parameters of specificity, accuracy, precision, linearity, and sensitivity, the method under validation has proved to be suitable for the analysis and quantification of HB in the range of 0.5–25 µg/mL in ex vivo permeation studies using human skin.

2.2. Skin Permeation Assay and Permeation Parameters

HB formulation permeations were performed on human skin for a period of 24 h, following the methodologies described and complying with the guidelines [32–35]. Transepidermal water loss (TEWL) measurement was performed on all skin pieces, and the values obtained were indicative of a stratum corneum in a condition fit for using in ex vivo permeation tests [36]. Samples were analyzed, and the permeation parameters were determined [37], along with the determination of retained amount of HB per gram of skin. As Transcutol® is a permeation enhancer, a blank solution was used with the same drug concentration, but without any other promoter. For the calculation of the permeation parameters such as flux (J) and permeability coefficient (Kp), a plot of the cumulative amount of drug permeated versus time was made. It can be seen that HB has a better permeation profile in the presence of menthone and nonane compared to the other permeation enhancers studied (Figure 2).

The permeation of HB obtained in the presence of Transcutol® tends to zero in comparison to other enhancers studied. Comparing the drug fluxes in the presence of each promoter, HB permeates 18 times faster in the presence of menthone in comparison to nonane, and on average, 30 times faster than the other enhancers under study, obtaining the lowest flux in the presence of azone (Table 4).

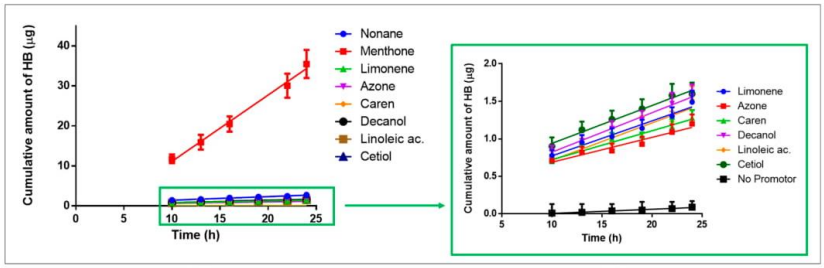


Figure 2. Cumulative permeated amount of HB versus time (h) represented as mean ± SD of six experiments. (**a**) Effect of permeation enhancers in the permeation of HB through skin; (**b**) zoom of the enhancers with lower effect in HB permeation and HB with Transcutol® (No promotor).

Table 4. Skin permeation parameters of HB in the presence of tested promoters. Data are represented as median (min–max).

Permeation Enhancer	J (µg·h^{-1} cm^{-2}) (min–max)	Kp (cm h^{-1})·10^5 (min–max)	A_{24} (µg)	As (µg·g^{-1} cm^{-2}) (min–max)
Nonane	0.141 ^{b,c,d,e,f,g,h} (0.138–0.167)	14.1 ^{b,c,d,e,f,g,h} (13.8–16.7)	2.74 ^{b,c,d,e,f,g,h} (2.57–3.82)	302.70 ^{b,c,d,e,f,g,h} (280.04–315.76)
Menthone	2.588 ^{a,c,d,e,f,g,h} (2.476–2.734)	25.9 ^{a,c,d,e,f,g,h} (248–273)	35.47 ^{a,c,d,e,f,g,h} (31.84–9.27)	214.04 ^{c,d,e,f,g,h} (203.06–226.87)
Limonene	0.073 (0.061–0.078)	7.29 × 10^{-5} (6.10–7.81)	1.49 (1.40–1.77)	62.62 ^{d,e,f,g,h} (55.57–68.34)
Azone	0.052 ^{f,g} (0.046–0.056)	5.19 ^{f,g} (4.60–5.56)	1.2 (0.93–1.55)	71.17 ^{e,g,h} (65.75–76.45)
Carene	0.060 ^{f} (0.059–0.064)	6.03 ^{f} (5.90–6.40)	1.28 (1.09–1.61)	41.39 ^{f,g,h} (36.57–45.02)
Decanol	0.082 (0.077–0.087)	8.22 (7.70–8.70)	1.57 (1.17–1.78)	74.85 ^{g,h} (69.86–78.85)
Linoleic acid	0.078 (0.073–0.087)	7.79 (7.30–8.70)	1.52 (1.22–1.72)	24.74 (18.76–26.02)
Cetiol	0.071 (0.063–0.075)	7.06 (6.30–7.50)	1.6 (1.31–1.73)	22.04 (18.56–24.98)

Letters represent statistical significate differences ($p < 0.05$) ^{a} Nonane; ^{b} Menthone; ^{c} Limonene; ^{d} Azone; ^{e} Carene; ^{f} Decanol; ^{g} Linoleic acid; ^{h} Cetiol. Permeation parameters: flux (J); permeability coefficient (Kp); amount permeated at 24 h (A_{24}); amount of drug permeated (As).

The Kp is calculated from the division of the flux in the initial concentration of the sample (1 mg/mL) so that the differences between the Kp follow the same order as the flux differences. Under 24 h of study, the amount of drug permeated (A_{24}) was 35.47 and 2.74 μg for menthone and nonane, respectively. No significant differences were found between the other promoters, and the average permeated amount of drug was 1.45 μg (Table 4). From the results obtained from the extraction of the drug in the skin, we can see that the retention at 24 h of nonane (As = 302.70 $\mu g \cdot cm^{-2}\ g^{-1}$) is approximately 1.5 times greater than menthone (As = 214.04 $\mu g \cdot cm^{-2}\ g^{-1}$). The lowest retention was obtained in the presence of linoleic acid and cetiol (13 times lower than nonane). In spite of finding significant differences in the As of all enhancers (except between linoleic acid and cetiol), the greatest differences are seen when comparing nonane and menthone with all other promoters.

2.3. In Vivo Draize Skin Test

The Draize test is used to determine whether or not a substance is an irritant based on the appearance of erythema or edema. As menthone and nonane showed significantly greater effect in enhancing the human skin permeability of HB during ex-vivo experimentation, they were selected for further in-vivo studies. Their in vivo irritant potential effect was assessed in rabbits. For the formulations under study, no edema or erythema formation was found after 24 h of exposure. The individual primary irritancy index determined in three rabbits for each formulation was "0" for both, erythema and edema. Therefore, the formulations HB–menthone and HB–nonane are classified as nonirritant [38].

2.4. Efficacy Assay

The histamine wheal suppression test is used to determine the anti-inflammatory efficacy of a corticosteroid by forming a bleb when injecting intradermal histamine. It has been described that the maximum effect can be seen between 10 and 30 min post injection [39]. The wheal size was studied in the presence of HB, with and without permeation enhancers. The control corresponds to the bleb formed by histamine in the absence of HB. Figure 3 shows the inflammation produced in the back of the rabbit after 30 min, in which it is observed that the smallest and largest wheal formed corresponds to treatment with HB–menthone and the control, respectively. It also shows a slight redness in the control (d), which is absent in the treated groups (a, b, c), indicating that in the presence of menthone, the response effect is higher.

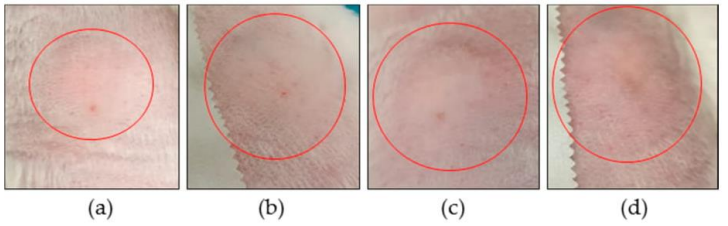


Figure 3. Photographs of histamine wheal suppression test at 30 min. (**a**) HB–menthone; (**b**) HB–nonane; (**c**) HB (in Transcutol®); (**d**) control without HB. Red circles indicate wheal formed.

Figure 4 shows the mean of the results. It is evident that in all sampling times, the formulation HB–menthone presents a smaller reaction to the test. This result agrees with ex vivo assays in which menthone was the permeation enhancer with the highest result of HB permeated.

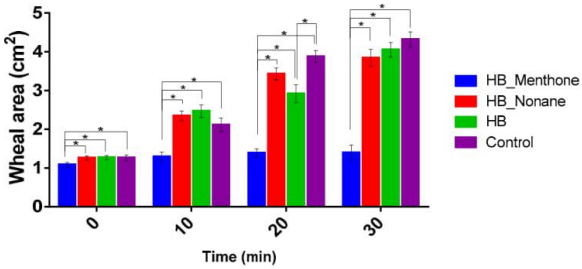


Figure 4. Results of histamine wheal suppression test are represented as mean ± SD. Significant differences are represented with * ($p < 0.005$).

3. Discussion

3.1. HPLC Validation Methodology

When making a formulation, it is necessary to count on a methodology that allows a reliable quantification of the drug during ex vivo assays. The skin is a complex matrix, and when used during experimentation, its components may interfere with the analytical process, and so may the other components of the formulations under study. This is the reason why a simpler, and easier methodology was developed, with less chemical reagents and at a low cost, in comparison to that provided by pharmacopoeia and other authors for the determination of HB [40]. Numerous methodologies have been described for the detection and quantification of HB in commercial formulations, including high performance liquid chromatography (HPLC), ultra performance liquid chromatography (UPLC) and spectrophotometric techniques [41–45], but so far, no methodology for the quantification of HB samples from ex vivo experimentation with biological tissues has been determined. The HPLC methodology for the determination of HB described by united states pharmacopoeia (USP) [40] uses gradient conditions. It takes approximately one hour to analyze each sample (retention time), and is designed for the quantification of HB without biological matrix, that is to say, without possible biological interferences. In this work we have optimized the methodology described by the USP to perform the analysis of the samples in 7–8 min with an isocratic acetonitrile/water flow which is easier and eight times faster. HB is practically insoluble in water (Cs < 0.007 mg/mL) [46], and most of the techniques described for the determination of non-biological complex samples need pre-treatment, and have to be solubilized in water or a buffer, and this procedure could alter the drug stability [43,45]. Our analytical methods avoid these steps and their related difficulties. Transcutol® is a solubilizer generally used in formulations for skin [47], and delivers a chromatogram with initial peaks that are maintained once it has crossed the barrier. The differences in these initial peaks would be determined by the presence of the promoter used in each formulation, and the presence of some element of the matrix capable of being solubilized with Transcutol®.

The validation of a method aims to demonstrate that it is suitable for the purpose it is being used for. Therefore, this analytical method has been validated considering its application and field of use. The parameters of linearity, precision, accuracy, selectivity, and sensitivity have been determined as required by International Conference Harmonization Guidelines (ICH) in the range of 0.5–25 µg/mL, when HB detection is required from samples obtained by ex vivo permeation studies in human skin.

3.2. Ex Vivo Studies

Skin is divided into layers, the composition of each one of which varies with the depth at which it is analyzed. The lipid composition of the skin includes sphingolipid, polar, neutral, and apolar lipids among others, which are affected by the promoters when they are used to modulate the permeation of a drug [48]. For a drug to penetrate through the skin, three possible routes are suggested: polar, apolar,

or a mixture of both. Although the mechanism of the enhancers' action is not fully explained, it has been described that they act in altering some of these pathways.

A polar alteration is given by the generation of a conformational change or denaturation of the proteins. An apolar alteration is a consequence of the fluidization of the lipid structure that increases the passive diffusion of a drug [29,49]. Enhancers can also alter the partition coefficient of the drug, K_m and the permeability of the skin, while maintaining its integrity. Generally, they present at least two simultaneous mechanisms of action [26,29]. Transcutol® is a solvent and drug solubilizer that can act as a permeation promoter with a low toxicity [47]. This solvent was selected due to its ability to solubilize the drug. The results of the permeation profiles indicate that Transcutol® would not act as a permeation accelerator for HB. This result is similar to that found by Bonina & Montenegro [50] in which it was described that for sodium heparin, Transcutol® has no effect as a permeation enhancer. Therefore, Transcutol® was used only for solubilizing the drug and not as a permeation promoter. The permeability enhancers selected for this study have been previously investigated by other authors [1,4,24,26], who obtained varying results regarding the enhancers' effectiveness, depending on the drug used. They were selected based on their ability to modulate lipophilic compounds. It is known that the action of an enhancer is drug-specific because of the chemical characteristics of both, the enhancer and the drug. Azones have been widely studied: it is believed that their mechanism of action is to act as a solvent of the lipids of the skin, denaturing proteins and modifying the drug diffusion coefficient in the process of permeation through the skin. Several studies have been conducted in which azone increases the permeation of compounds such as antifungal, antimicrobial, triamcinolone, and other corticosteroids, reporting in some cases, irritation problems [26,51,52]. The alkanes exert their promoter effect by being slightly irritating, extensively altering the stratum corneum, and therefore, the barrier function of the epidermis. This increases the permeation of the drugs without damaging the skin [53]. Its activity has been described in drugs such as propranolol hydrochloride [54,55]. On the other hand, fatty acids have been reported to increase drug permeability by causing disturbance of the intercellular lipid bilayer present in the stratum corneum, and that its promoter effect may be influenced by branching and chain length [56]. Fatty acid promoters have been shown to be effective in increasing corticosteroid penetration in betamethasone 17-benzoate [57,58] and hydrocortisone [59].

The highest permeation obtained from HB for all sampling times was in the presence of menthone, a promoter belonging to the terpenes family. The mechanism of action of this promoter has not been fully studied, but it is believed to cause a reversible alteration of skin lipids, thus helping the permeation of anti-inflammatory drugs with a cholesterol-like base structure [31,49,60]. Limonene and carene are hydrocarbon terpenoids, whereas menthone is a ketone terpenoid [61]. This difference could explain the fact that although all three are terpenes, menthone exerts a higher permeating effect. The same may occur with alkanes, on which nonane has a better permeating effect than decanol, due to the differences in the chain length. Because of this, there is the possibility that despite belonging to the same chemical group, not all enhancers produce the same result with a particular drug. When modulating the permeation of drugs such as corticosteroids, it is necessary to consider the possible systemic effect that they have, so that adverse effects can be ruled out, or lessened. The promoters that increased the permeation of HB in descending order were: menthone, nonane, cetiol, decanol, limonene, linoleic acid, careen, and azone. The highest HB permeated amount was 35.47 µg, and the lowest was 1.20 µg at 24 h. The enhancers that allow major retention of HB in skin after 24 h, in descending order, were nonane (302.70 $\mu g \cdot g^{-1}$ cm^{-2}), menthone, decanol, azone, limonene, carene, linoleic acid, and cetiol (22.04 $\mu g \cdot g^{-1}$ cm^{-2}).

3.3. In Vivo Studies

The skin tolerance and efficacy tests were performed on rabbits. Although it has been described that their skin may be more permeable to certain compounds, they are animal models which are easy to handle, inexpensive, and they have human-like skin characteristics, all of which make them a good approximation prior to the human determinations [62–64]. The Draize test was negative for

erythema and edema after 24 h of exposure to HB formulations with menthone and nonane. The drug, Transcutol®, and the promoters used, are approved by the food and drug administration (FDA) and can be used in commercial formulations [10], so it was expected that they would not cause any skin alterations in the proportions used.

Several methodologies have been described to determine the efficacy and penetration of topical corticosteroids. Among them, we have the vasoconstriction test, radiolabeling, and micro dialysis [65–67]. The histamine-induced wheal suppression test performed has the advantage of being a simple, reliable, reproducible, and non-invasive method compared to other techniques. This test can also mimic the inflammatory conditions such as redness and temperature where a topical corticosteroid would be used. It can be performed using fewer animals per experiment, and allows the study of more than one substance in the same individual, if necessary [39]. After the ex vivo study, it was decided to choose the two formulations with higher permeation profiles in 24 h. HB–menthone and HB–nonane were selected for their in vivo efficacy test. The anti-inflammatory effect was higher for HB–menthone, followed by HB–nonane, both with better effect than the control. These results agree with those obtained in the ex vivo studies, in which HB–menthone exhibited a higher level of permeated HB than HB–nonane, as well as a higher flux. The developed HB–promoter formulations possess an euthermic pH, a characteristic odor, and a clear color, with a pleasant feel on touch. While they have not been tested in humans, they can be predicted to be safe. The maximum recommended dose of HB in commercial formulations should not exceed 50 g/week of a 0.05% HB cream or lotion with a maximum of two weeks treatment period. This corresponds to a maximum dose of 25 mg applied in two weeks (1.8 mg HB/day), to avoid inhibition of the hypothalamic–pituitary–adrenal axis, and other systemic effects [17,19,20]. According to the literature [20], about 6% of the applied dose reaches systemic circulation. The formulation HB–menthone presented a permeated amount of approximately 40 µg/day from a 0.1% formulation with a surface area of 0.64 cm^2, which is within the parameters established.

One limitation of this study is that ex vivo permeation tests are performed on healthy skin (due to confidentiality agreements, it is not possible to know if the donor had healthy or diseased skin beyond the TEWL test). In the future, research should test formulations on diseased human skin (psoriasis, dermatitis), considering the differences of permeation in diseased skin and the metabolic effects, if there are any. Thus, it would be possible to determine which formulation would be more suitable for each specific disease and patient, according to the disease characteristics and the patient's conditions.

4. Materials and Methods

4.1. Materials

HB was purchased from Capot Chemical Company Limited (Hangzhou, China). The permeation enhancers carene ((+)-3-carene), cetiol (decyltetradecyl ethylhexanoate), decanol (decyl alcohol), limonene ((*S*)-4-isopropenyl-1-methyl cyclohexene), linoleic acid (9-*cis*,12-*cis*-linoleic acid), menthone ((2*S*,5*R*)-2-isopropyl-5-methylcyclohexanone) and nonane (*n*-nonane) were acquired from Sigma Aldrich (Madrid, Spain) and Azone® (1-dodecylazacycloheptan-2-one) from Durham Pharmaceuicals (Durham, UK). Transcutol® (diethylene glycol monoethyl ether) was kindly given by Gattefossé (Barcelona, Spain). MilliQ Plus system was used to obtain purified water. All the other chemical reagents used were of analytical grade.

4.2. Methods

4.2.1. HPLC Validation Methodology

The HPLC equipment used for the analysis of these experiments was a Waters® Alliance 2695 separation module with Kromasil® C18 (5 µm, 15 × 0.46 mm) column (Technokroma, Barcelona, Spain). The mobile phase of acetonitrile/water (63:37) was under isocratic elution at a flow rate of 0.8 mL/min.

A diode array detector Waters® 2996 at a wavelength of 238 nm was used to detect HB. A volume of 50 µL of sample were injected. Data was processed using Empower 3® Software.

For the preparation of stock solution, 1.25 mg of HB were weighed out and dissolved in 25 mL of Transcutol®. A mixture of Transcutol®/water (T/w) 70:30 (*v*/*v*) was used for the preparation of the standards at concentrations of 0.5–1.0–5.0–10.0–15–20–25 µL/mL. The same solvent mixture was used as a blank. Five independent calibration sets were prepared, each one on a different day.

The validation process of this methodology was performed according to the ICH guidelines [68] The parameters of specificity, accuracy, precision, linearity, range, detection limit, and quantification limit were analyzed.

Specificity is defined by the ICH guidelines as the capability to assess, without mistake, the analyte under evaluation in the presence of other expected components. Depending on the samples and their treatment, these components may include matrix components, impurities, and reagents or solvents used in the process as required by the method. Specificity was assessed by the absence of interferences at the same retention time at which the analyte appears.

Accuracy is the ability of an instrument to approach the real value in a measurement. This parameter was calculated as follows:

$$\%RE = ((Co - Cn)/Cn) \quad (1)$$

where Co corresponds to observed concentration; Cn corresponds to nominal concentration and %RE represents mean percentage deviation (% Relative Error). For the assessment of accuracy, five calibration curves were prepared on different days.

Precision is the ability of an instrument to replicate a measurement with minimal variation and is expressed as the mean coefficient of variation (%CV). For the determination of this parameter, five standard curves were prepared.

Linearity is when, in a given range, the result obtained is directly proportional to the amount of analyte present in the sample. Five calibration curves were evaluated for linearity, each one with seven concentration levels. This parameter was evaluated by the determination coefficient (r^2) obtained from the analysis of least-squares linear regression of the calibration sets. The processing of the data was using MS excel software (Microsoft Corporation, Redmond, WA, USA). Linearity was also determined by one-way analysis of variance (ANOVA) test, comparing the concentration of a tested standard with the ratio of the areas by Graph Pad Prism® (version 6.01, Graph Pad Prism software, Inc., La Jolla, CA, USA)

The range represents the difference between the maximum and minimum values, and uses the same units as the data. The range is obtained according to the linearity studies, and indicates the concentration at which it has been proved that the method has a suitable level of linearity, accuracy and precision.

To define sensitivity of an analytical method, it is necessary to determine the limit of detection (LOD) and the quantification limit (LOQ). The first of these is defined as the minimum amount of analyte that can be detected, but not necessarily accurately quantified. The second LOQ is the minimum concentration than can be accurately quantified with acceptable accuracy and precision. The acceptable limits for precision and accuracy for calibration standards were set at 20% CV and ±15% respectively. The determination of LOD and LOQ in accordance with the ICH guidelines were estimated based on a standard deviation of the slope and the response, as follows:

$$LOD = [(3.3\ s)/\rho] \quad (2)$$

$$LOQ = [(10\ s)/\rho] \quad (3)$$

where "s" represents standard deviation of the Y-intercept, and "ρ" represents the slope on the calibration curve.

4.2.2. Ex Vivo Assays

HB is a lipophilic and highly insoluble in water corticosteroid [46,69]. In the light of this, the formulations were prepared diluting the drug in Transcutol® (to meet the Sink conditions, Cs > 20 mg/mL) and 5% v/v of each promoter in order to obtain a final concentration of 1.0 mg/mL HB in each sample solution.

Skin Permeation Assay

Human skin from the abdominal region was obtained from a healthy woman during plastic surgery and was used as a permeation membrane (Barcelona-SCIAS Hospital, Barcelona, Spain). The volunteer gave written informed consent and the experimental protocol was approved by the Bioethics Committee of the Barcelona-SCIAS Hospital. For the drug permeation study, a dermatome (Model GA 630, Aesculap, Tuttlingen, Germany) was used to cut a piece of skin with a thickness of 0.4 mm [35,70]. With the stratum corneum facing the donor compartment, the skin was placed between the donor and receptor compartment in a Franz-type cell [71] (Hanson Research, Chatsworth, CA, USA; Crown Glass Company, INC, Jersey City, NJ, USA) with a 0.64 cm^2 diffusion area, and fixed with paraffin film to prevent it from leaking. T/w (70:30) was used as a receptor solution in the receptor chamber. The cells were connected to a controlled bath at a temperature of 32 °C throughout the complete experiment. The skin was allowed to equilibrate for 30 min prior to the application of the formulation, and skin barrier integrity was assessed by measuring transepidermal water loss (TEWL) (TEWL-meter TM210, Courage & Khazaka, Koln, Germany). The probe was placed on the donor compartment in close contact with the skin, and lightly pressed to record the skin moisture content. Human skin pieces exhibiting TEWL values below 10 $g/m^2 \cdot h$ were used [72]. One milliliter of each formulation was placed onto the donor compartment in contact with the epidermal side of the skin. At a given time interval, 300 µL of sample from receptor compartment were withdrawn and replaced with the same volume of receptor solution, until there had been 24 h of contact. The amount of drug permeated was determined using HPLC and permeation parameters such as flux (J), permeability coefficient (Kp), amount permeated at 24 h (A_{24}), and the drug amount retained (As) in skin was calculated using a linear least-squares regression model with GraphPad Prism® (version 6.01, GraphPad Prism software, Inc.) software. All samples were made by using skin from the same donor, so as to diminish the variability of the response due to biological differences.

Amount of Drug Retained in Skin

Once the permeation study was finished, the skin was removed from the cell and used to determine the amount of drug retained using the protocol described elsewhere [73]. The skin was cleaned with a gauze soaked in solution of 0.05% of sodium dodecyl sulfate and distilled water to remove the excess of formulation on the surface [74]. The diffusional area of the skin in contact with the formulation was isolated and weighed. For the drug extraction, the skin was punctured with a needle, placed in a vial with 1 mL of acetonitrile and sonicated during 20 min at room temperature. The solutions obtained were measured directly by HPLC, indicating the amount of drug retained in the skin expressed in ($\mu g \cdot g^{-1}$ cm^{-2}). Nonparametric Mann–Whitney statistical tests were performed to compare drug retention from different formulations [37].

Recovery Percentage

For this determination, a piece of skin of the same donor was weighed and placed in a tube with 1 mL of solution with a known concentration of HB in Transcutol®. The tube was kept in a bath for twenty-four hours at a temperature of 32 °C. After this time, the skin was removed, and the supernatant was measured by HPLC to determine the amount of drug that had penetrated. The amount of drug

retained in the skin was determined using the same extraction procedure previously described for the samples. The recovery percentage was calculated as follows:

$$\text{Recovery (\%)} = \text{Amount retained}/\text{Amount penetrated} \times 100 \quad (4)$$

This recovery percentage was used to calculate and correct the amount of HB retained in samples.

4.2.3. In Vivo Assays

Two in vivo tests were conducted in order to evaluate the tolerance and the efficacy of the drug. The Draize skin test was performed on male albino rabbits of 1.9–2.0 kg weight following the current international guidelines [35] to determine skin tolerance. Approval was obtained by the Animal Research Ethical Committee of the University of Barcelona, according to the regulations of the local government (Decree 214/1997, 30 July). Twenty-four hours before the test, the rabbit's back was shaved with an electric razor, revealing two squares of 5 × 5 cm each, and scarred with a lancet. Then, 0.5 mL of HB–promoter solution was applied on each square, and the site was left uncovered until the next day. In accordance with the principles of 3R (reduction, refinement, and replacement) only three animals were used for each HB formulation [74]. After 24 h of exposure to the selected formulation, the excess was removed, and the skin was scored for edema and erythema (both graded from 0 to 4). The individual primary irritancy index was determined for each rabbit, adding the edema and erythema scores based on a standard scale. The mean value of the three rabbits' scores was calculated. Taking into account the primary irritancy index value, they were classified as "nonirritant" (0–0.5), "mildly irritant" (0.5–2) "moderately irritant" (2–5), or "severely irritant" (5–8) [38].

The histamine-induced wheal suppression test was performed in order to compare the efficacy of the formulations with the best kinetic parameters [39]. In the procedure, New Zealand male albino rabbits of 1.9–2.0 kg were used. The animals were kept in standard cages with food and drink ad libitum. The day prior to the experiment, the rabbits' back hair was shaved with an electric razor, avoiding harming their skin. The left side was used as a control and the right side as a treatment zone. 0.5 mL of the HB formulation (0.05%) was applied on the treatment side, and 0.5 mL of Transcutol® without drug was applied on the control side. One hour later, the excess of formulation was removed with cotton wool and the histamine test was performed. A solution of histamine dihydrochloride (0.05 mL of 0.1% in distilled water) was injected intradermally with an insulin syringe. The size (cm^2) of the bleb generated by the injection was measured with a caliper at 10, 20, and 30 min. Statistical analysis was made using GraphPad Prism® (version 6.01 GraphPad Prism software, Inc.) software.

5. Conclusions

Based on the results obtained, it can be concluded that the presence of different permeation enhancers allows modulating permeation profiles, and thus, they affect the drug's efficacy. We can highlight that for a formulation with HB, menthone and/or nonane are the most relevant permeation enhancers to increase the permeation and retention of the drug in the skin, respectively. Therefore, HB formulations containing menthone or nonane are suggested as prototypes for further clinical assays focused on the treatment of skin inflammatory diseases, such as, for instance, psoriasis and atopic dermatitis, among others. Due to the characteristics of the formulations, they could be used in pharmaceutical dosage forms such as roll-on or a flask with dosing cap.

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3.2. Development of Halobetasol-loaded nanostructured lipid carrier for dermal administration: Optimization, physicochemical and biopharmaceutical behavior, and therapeutic efficacy.

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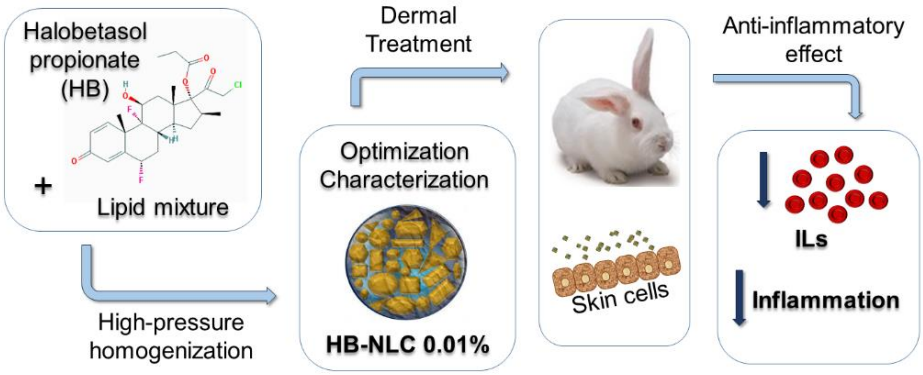

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Development of Halobetasol-loaded nanostructured lipid carrier for dermal administration: Optimization, physicochemical and biopharmaceutical behavior, and therapeutic efficacy

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Abstract

Halobetasol propionate (HB) is considered a super potent drug in the group of topical corticosteroids. HB has anti-inflammatory activity, vasoconstriction properties, and due to its high skin penetration, it can cause systemic side effects. To improve its characteristics, enhance topical effectiveness and reduce penetration to systemic circulation, a study to optimize and characterize a HB-loaded lipid nanocarrier (HB-NLC) has been made by high-pressure homogenization method. The formulation is composed by HB, surfactant, glyceryl distearate and capric glycerides. The optimized HB-NLC containing 0.01% of HB and 3% of total lipid shows an average size below 200 nm with a polydispersity index $\ll 0.2$ and an encapsulation efficiency $\gg 90\%$. The *in vitro* and *in vivo* tests indicate that the HB-NLC is not toxic, is well tolerated and has an anti-inflammatory effect because they decrease the production of Interleukins in keratinocytes and monocytes. HB-NLC is considered an alternative treatment for skin inflammatory disorders.

Keywords: Halobetasol; Corticosteroids; Lipid nanocarrier; Skin diseases; Inflammation

Inflammatory processes are an innate and non-specific skin responses towards aggressions or external environment changes. Skin is the largest organ of human body, and under certain conditions, it can suffer varied alterations and diseases which main characteristics are edema, inflammation, temperature increase and redness. Diseases that commonly occur with inflammation are atopic dermatitis, vitiligo, psoriasis and eczema.[1] For these sort of skin illnesses, topical steroids are generally used.[2,3] These drugs, despite being very effective, can produce severe topical and systemic side effects such as stretch marks, pigmentation, skin thinning and Cushing Syndrome when used for long periods of treatment.[4,5] Topical corticosteroids can be classified according to their potency into four groups (Class I-IV), being Class I the most potent and Class IV the least. Drugs as Clobetasol and Halobetasol propionate (HB) belong to Class I, called "super-potent" corticosteroids. Their anti-inflammatory action mechanism consists in modulating the expression of certain genes in order to produce an increase in anti-inflammatory proteins and inhibit the release of some substances involved in pro-inflammatory responses such as cytokines and

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adhesion molecules, among others.[6] One alternative to modify the release of this type of drugs and to reduce the adverse effects, considering the properties of the skin and the characteristics of the drug, are the nanostructured lipid carriers (NLC).[7,8] It has been proven that these systems allow modulating the penetration/permeation of drugs through the different layers of the skin.[9] The NLCs are the second generation of lipid nanoparticles and their main characteristic as well as its advantage, is to be composed by a mixture of liquid lipid (oil) and solid lipid that generates an imperfect crystalline matrix which allows the solubilization and incorporation of the drug into the lipid mixture.[10] Among the most used lipids for the generation of these NLC are triglycerides, fatty acids, waxes, oleic acid and vegetable oils, lipids considered bio-compatible and therefore, safe to use.[11–13] HB is a synthetic and highly lipophilic drug with anti-inflammatory, anti-pruritus and vasoconstrictor activity used in skin diseases such as psoriasis and atopic dermatitis.[2] Notwithstanding its effectiveness, due to its possible local and systemic side effects, is not recommended their use, for more than 2 weeks.[14] Because of the described above, the encapsulation of HB in a NLC system would be a good alternative to modulate the release of the drug and decrease the systemic side effects. The primary objective of this study was to develop a new dermal formulation of HB loaded NLC (HB-NLC), composed of glyceryl distearate and capric glycerides. Optimization, physical characterization, short-term stability and *in vitro/ex vivo* (human skin) biopharmaceutical behavior tests were carried out. Tolerance and *in vitro/in vivo* efficacy tests were also performed to demonstrate the formulation suitability as a new and safer alternative for the treatment of inflammatory skin diseases.

Methods

Materials

HB (21-chloro-6α,9-difluoro−11β,17-dihydroxy−16βmethyl-lpregna−1,4-diene-3-20-dione,17-propionate) and Tween®80 (Polysorbate 80) were purchased from Capot Chemical Company Limited (Hangzhou, China) and Sigma Aldrich (Madrid, Spain) respectively. Solid lipids and liquid lipids tested (S1) were obtained from Sigma Aldrich (Madrid, Spain) and kindly gifted from Gattefossé (Madrid, Spain). All other chemical reagents and components used were of analytical grade, a Millipore Milli-Q Plus system was used to obtain purified water.

HB-NLC preparation and optimization

Based on a list of suitable lipids (solid and liquid) for topical administration, a screening was realized to determine the drug solubility per gram of lipid (S1).[15] Based on drug solubility, a solid lipid and a liquid lipid were selected to produce HB-NLC by high-pressure homogenization method (Homogenizer FPG 12800, Stansted, United Kingdom)[16,17] after generating a primary emulsion with the mixture of components with an Ultraturrax T25 (IKA, Germany) at 8000 rpm for 30 s. Ratio liquid/solid lipid (20:80) was kept constant (S2) and the production conditions were 80 °C and three homogenization cycles (S3).

A design of experiments (DoE) was performed in order to optimize formulation parameters.[18] A central composite factorial design 2^3 (containing 2 replicated center points, 8 factorial points and 6 axial points) was developed using statistical program Statgraphics Centurion XVI.II® version 16.2.04 software (Virginia, USA). Three independent variables: HB concentration (cHB mg/ml), surfactant concentration (cTw mg/ml) and lipid percentage (cLipid %) were evaluated to determine their influence on the NLC properties. Mean particle diameter size (Z_{av}), Polydispersity index (PI), entrapment efficiency (EE) and Zeta potential (ZP) were designated as the dependent variables.

Z_{av} and PI were assessed by photon correlation spectroscopy (PCS) with a ZetaSizer Nano ZS (Malvern Instruments, Malvern, UK). ZP was estimated by electrophoretic mobility.[19] For these measurements, samples were diluted with Milli-Q water 1:20 and analyzed thrice at 25 °C. EE was indirectly determined by diluting the HB-NLC suspension in water and separating the non-encapsulated drug by filtration/centrifugation. HB amount contained in HB-NLC was measured by high-performance liquid chromatography (HPLC) method described previously.[20] EE was calculated using the equation:

$$EE(\%) = \frac{(\text{total amount of HB-free amount of HB})}{\text{Total amount of HB}} 100 \qquad (1)$$

Characterization of optimized HB-NLC

Transmission electron microscopy (TEM) was used to determine HB-NLC morphology with a JEOL 1010 microscope (Akishima, Japan). A drop of the formulation was added on a copper grid coated with carbon film and negatively stained with 2% of phosphotungstic acid.[21]

X-ray spectroscopy (XRD) was used to analyze the amorphous or crystalline state of the samples (centrifuged and dried NLC and solid components). Samples were sandwiched between 3.6 μm films of polyester and exposed to CuKα radiation (45 kV, 40 mA, λ = 1.5418 Å) in the range (2θ) of 2° to 60° with a step size of 0.026° and a measuring time of 200 s per step.[22]

Fourier transform infrared (FTIR) spectra of components and HB-NLC were obtained using a Thermo Scientific Nicolet iZ10 with an ATR diamond and DTGS detector. The scanning range was 525–4000 cm^{-1}.[22]

Differential scanning calorimetry (DSC) analysis were acquired using a Mettler TA 4000 system (Greifensee, Switzerland) equipped with a DSC 25 cell. The samples were directly weighted in a closed aluminum pan (Mettler M3 Microbalance) and heated up from 25 to 85 °C under nitrogen flow at a rate of 5 °C/min. An empty pan with the same characteristics was used as reference.[23]

Stability studies

HB-NLC were stored at 4, 25 and 37 °C during 3 months. The study was assessed analyzing light backscattering (BS) profiles by a Turbiscan® Lab equipment. A glass measurement cell containing 20 ml of sample was used. Data were acquired at days 1, 10, 30, 60 and 90. The radiation source used was pulsed near-

infrared light-emitting diode LED (λ = 880 nm), the signal was detected by a BS detector at an angle of 45° from the incident beam. At the same time interval, values of Z_{av}, PI, ZP and EE were measured.

Biopharmaceutical behavior

The *in vitro* HB release test for HB-NLC was performed using Franz-type diffusion cells (Crown Glass, NY, USA) with a diffusion area of 2.54 cm^2 and dialysis membranes of cellulose (MWCO 12 kDa). A solution of Transcutol®/water (70:30) under continuous stirring was used as a receptor medium assuring sink conditions. The HB-NLC formulation was compared with free-HB solution. The assay was carried out at 32 ± 0.5 °C along 52 h. 300 µl of the HB-NLC formulation were added to the donor compartment by direct contact with the membrane. At a certain time interval, 300 µl of sample were collected with a syringe and the volume withdrawn was replaced with receptor solution. Drug content of the samples was analyzed with HPLC. The test was performed thrice and the cumulative amount of HB was calculated.

Ex vivo skin permeation test was performed. Human skin from a healthy woman (abdominal region) was used as a permeation membrane. The skin was obtained from the Barcelona-SCIAS Hospital, (Barcelona, Spain) using a protocol approved by the Bioethics Committee of the Barcelona-SCIAS Hospital during a plastic surgery procedure. Permeation study was assessed following a methodology described elsewhere.[19] Briefly, a piece of skin of 0.4 mm of thickness,[24] with stratum corneum facing the donor compartment was placed in a Franz-type cell[25] (Crown Glass, NY, USA) between donor and receptor chambers at 32 °C in a controlled bath temperature. At a certain time interval, 300 µl of sample were withdrawn and replaced with receptor solution (Transcutol®/water 70:30) until reach 24 h of contact. The samples were directly measured by HPLC.

Cytotoxicity and skin tolerance

In vitro MTT cytotoxicity assay[26] was used to determine human keratinocytes (HaCaT) and human monocytes (THP-1) viability. Cells were incubated with different HB-NLC concentrations from 0.05 to 12.85 µM of HB for 24 h. The absorbance was read using a Microplate Autoreader at excitation/emission of 540/630 nm (Turner BioSystems). Negative control (cells without any stimulation or treatment) was processed for comparison. Absorbance values were considered proportional to cell viability, and the percentage cell viability was calculated using the following expression:

$$\text{Cell viability} = \left[A_{sample}/A_{control}\right] 100 \quad (2)$$

To assess *in vivo* skin tolerance, Draize skin test was used. New Zealand male albino rabbits of 2.0–2.2 kg weight were used following the current international guidelines.[27] The animals were kept under controlled ambient conditions with food and water *ad libitum*. The protocols used were approved by the Animal Research Ethical Committee of the University of Barcelona according to the regulations of the local Catalonian government (number 9246). Tests were carried out thrice.[28] The day before the experiment, rabbit's back hair was shaved, one side was scarred with a lancet and the other was left as a control. For the experiment, 0.5 ml of HB-NLC suspension was applied on the back and it was left uncovered. The excess of the formulation was removed after 24 h of contact, then, the skin was evaluated for edema and erythema (both graded from 0 to 4) based on Draize skin test score. This individual primary irritancy index allows classifying the substance as "nonirritant", "mildly irritant", "moderately irritant" or "severely irritant" taking into account the mean value score of the three rabbit's.[29]

Anti-inflammatory efficacy

In vitro cytokine determination was carried out in cells present in skin inflammatory processes. HaCaT and THP-1 cells were grown for 24 h and exposed, to the different concentrations of HB-NLC (0.13, 0.30, 0.50, 1.28, 2.57 and 5.14 µM of HB) in presence of TNFα (50 ng/ml) for HaCaT or LPS (10 ng/ml) for THP-1. HaCaT and THP-1 cells stimulated with TNFα or LPS without HB-NLC were taken as positive control, and cells without any stimulation as the negative control. After 24 h of incubation, supernatants were collected and Interleukin-8 (IL-8) was measured using the enzyme-linked immunosorbent assay (ELISA) following manufacturer's manual.

In vivo HB-NLC anti-inflammatory effectiveness was evaluated using the histamine-induced wheal suppression test in New Zealand male albino rabbits described previously. Two sides from the back of the rabbit were shaved, left side and right side were used as a control and treatment zone respectively. HB-NLC 0.5 ml were applied on the treatment site and a nanocarrier suspension without drug (NLC) was applied on the control site. After one hour, the excess of the formulation was removed and the test was performed. 0.05 ml of histamine dihydrochloride 0.1% solution were intradermally injected with an insulin syringe. At 10, 20 and 30 minutes the size of the bleb generated was measured with a caliper in cm^2.[30] Tests were carried out thrice.

Ex vivo gene expression analysis for Interleukin-6 (IL-6) and IL8 were determined in rabbit's back samples by RT-qPCR. Total RNA was extracted from small sections of rabbit's back tissue with and without HB-NLC treatment. Tissue was washed with PBS 1X and homogenized in 1 ml of ice-cold TRI Reagent® (Sigma Aldrich). Purity and RNA concentration were measured by the absorbance ration at λ 260 and 280 in a Thermo Scientific Nano Drop TM 2000 Spectrophotometer. RNA integrity was verified before mRNA quantification. Using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) under manufacturer's recommendations, reverse transcription was made. RT-qPCR reactions were achieved in a StepOne Plus PCR cycler (Applied Biosystems) by using SYBR® Green PCR Master Mix (Applied Biosystems) and specific oligonucleotides for IL-8, IL-6 and housekeeping GAPDH were used. Five replicates were made.

Statistical analysis

The statistical comparisons were developed using two-tailed t-test with Welch's test, significance of $\alpha \ll 0.05$. The normality and equality of variances were confirmed by F test. All analyzed data are presented as mean ± SD. GraphPad Prism® 6.01 software for windows was used to analyze the data.

Table 1
Design of experiments of HB-NLC.

	Independent variables						Dependent variables			
	cHB (mg/ml)		cTw (mg/ml)		cLipid (%)		Z_{av} (nm)	PI	ZP (mV)	EE (%)
Factorial points										
F1	-1	0.075	-1	0.15	-1	2.0	123.9 ± 2.8	0.154 ± 0.010	−19.8 ± 3.4	96.5 ± 0.3
F2	1	0.125	−1	0.15	−1	2.0	132.3 ± 2.1	0.195 ± 0.001	−16.8 ± 1.6	95.6 ± 1.8
F3	−1	0.075	1	0.19	−1	2.0	122.6 ± 1.8	0.148 ± 0.003	−21.1 ± 0.5	94.8 ± 0.5
F4	1	0.125	1	0.19	−1	2.0	135.4 ± 2.2	0.201 ± 0.005	−20.0 ± 0.2	95.5 ± 0.8
F5	−1	0.075	−1	0.15	1	3.0	143.4 ± 5.7	0.133 ± 0.001	−17.4 ± 0.6	95.8 ± 0.6
F6	1	0.125	−1	0.15	1	3.0	158.9 ± 6.2	0.167 ± 0.015	−16.6 ± 0.3	95.3 ± 0.3
F7	−1	0.075	1	0.19	1	3.0	144.2 ± 2.8	0.155 ± 0.001	−21.4 ± 0.1	95.1 ± 0.1
F8	1	0.125	1	0.19	1	3.0	147.1 ± 4.5	0.181 ± 0.013	−20.7 ± 0.2	94.9 ± 0.2
Axial points										
F9	1,68	0.142	0	0.17	0	2.5	149.7 ± 6.7	0.183 ± 0.001	−18.5 ± 0.1	95.8 ± 1.1
F10	−1,68	0.058	0	0.17	0	2.5	137.2 ± 3.7	0.146 ± 0.016	−20.8 ± 0.3	96.4 ± 0.2
F11	0	0.100	1,68	0.21	0	2.5	134.3 ± 3.3	0.163 ± 0.001	−19.7 ± 0.6	94.2 ± 0.9
F12	0	0.100	−1,68	0.14	0	2.5	136.1 ± 11.1	0.166 ± 0.008	−19.9 ± 0.5	94.5 ± 2.8
F13	0	0.100	0	0.17	1,68	3.3	167.1 ± 1.9	0.135 ± 0.013	−19.6 ± 0.2	94.9 ± 1.4
F14	0	0.100	0	0.17	−1,68	1.7	118.3 ± 8.1	0.180 ± 0.016	−19.9 ± 0.4	95.3 ± 0.7
Central points										
F15	0	0.100	0	0.17	0	2.5	136.1 ± 1.1	0.171 ± 0.003	−20.4 ± 0.3	93.7 ± 1.1
F16	0	0.100	0	0.17	0	2.5	141.0 ± 9.6	0.164 ± 0.012	−20.4 ± 0.1	95.1 ± 0.5

Results

Preparation and optimization of HB-NLC

Based on the drug solubility in different lipids, glyceryl distearate (Precirol®) as solid lipid and PEG-8 caprylic/capric glycerides (LAS) as liquid lipid were chosen to produce the HB-NLC. The Precirol®/LAS 80:20 ratio provides a homogenous and solid bulk mixture at room temperature that allows the solubilization of the drug and the development of NLC (S1). Table 1 shows the effect of independent variables used on dependent variables analyzed and the values obtained. Z_{av} values ranged between 100 and 200 nm. The developed HB-NLC exhibits a negative surface charge ZP ≪ −10.0 mv. PI values are ≤0.200 which indicates a monomodal distribution. In all cases EE was higher than 90%. As it can be appreciated in Figure 1, the three variables studied had a significant effect on the formulation of the NLC. Diameter of HB-NLC (Z_{av}) is directly influenced by cLipid and cHB (Figure 1, *A*). Higher lipid concentrations provide HB-NLC of larger size but with lower PI (Figure 1, *B*). ZP is influenced by the amount of surfactant, a higher concentration of surfactant increases (in absolute value) the superficial charge and gives more stability to the formulation (Figure 1, *C*) EE is significantly influenced by the cHB (Figure 1, *D*), but at the same time, this variable influences increasing all the parameters under study. With these trends, the optimized formulation contains 0.125 mg/ml of HB, 3% of total lipid and 19 mg/ml of Polysorbate 80.

Characterization of optimized HB-NLC

The morphology of the HB-NLC obtained by TEM (Figure 2, *A*) shows almost spherical and soft shapes and the size below 200 nm is consistent with the results founded by PCS. Particle aggregation phenomena is not observed.

XRD profiles in Figure 2, *B* show the physical state of HB incorporated in NLC. Intense and sharp peaks for HB and for the solid mixture of lipids are shown, indicating that these components have a crystalline structure. The peaks found for HB are not detected in HB-NLC profile, which could mean that the drug is present in a dissolved state in the NLC (molecular dispersion).[31]

FTIR spectra (Figure 2, *C*) shows the characteristic peaks of HB at 1609, 1625, 1662, 1708 and 1740 cm^{-1} corresponding to stretching vibration of C=O. Lipid mixture shows characteristics bands at 1470 and 1725 cm^{-1} due to stretching vibration of C=C and C=O respectively, two peaks between 3000 and 2800 cm^{-1} corresponding to stretching vibration of CH and one peak at 3480 cm^{-1} due to stretching vibration of OH.[32,33] HB-NLC spectra is similar to lipid mixture.

DSC was carried out in order to study the crystallinity and the melting point variations of the lipid mixtures and HB-NLC. Thermogram (Figure 2, *D*) shows endothermal peaks, of 52.5 °C for lipid mixture, 53.4 °C for lipid mixture-HB and 53.9 °C HB-NLC. The peaks move to higher temperatures when HB is added and the enthalpy decreases being ΔH Lipid mixture = −112.47 Jg^{-1}, ΔH Lipid mixture-HB = −109.53 Jg^{-1} and ΔH HB-NLC = −97.92 Jg^{-1}. HB melting transition is characterized by an endothermal peak at 219.1 °C (ΔH = 34.09 Jg^{-1}) followed by decomposition.[15]

Stability studies

BS profiles of HB-NLC at three different storage temperatures are shown in Figure 3. Apparently, the HB-NLC formulation is stable at 4 °C (Figure 3, *A*) for a period of 2 months, while at 25 °C (Figure 3, *B*) the stability endures only 1 month. The instability of the formulation is evidenced when BS profile presents differences greater than 10% regarding the initial profile.[23,34] At 37 °C there is a great variability from day 10 onwards. At 4 °C Z_{av} and PI are relatively maintained for 3

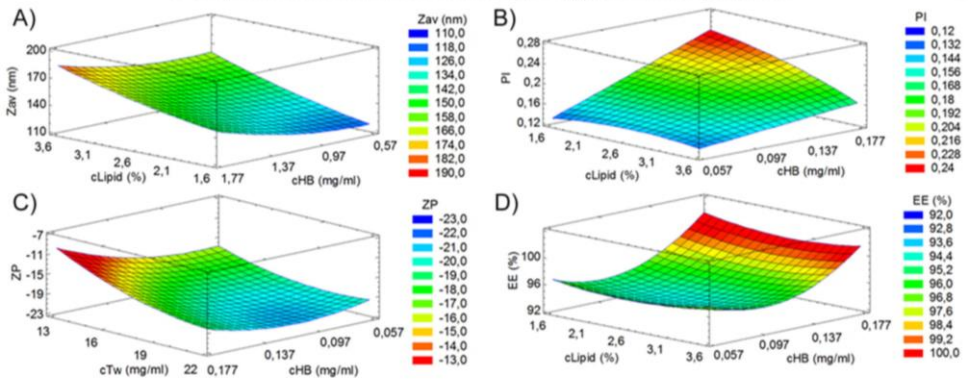


Figure 1. DoE surface response of HB-NLC. **(A)** cHB and cLipid influence on Z_{av}. **(B)** cLipid and cHB influence on PI. **(C)** cTw and cHB influence on ZP. **(D)** cLipid and cHB influence on EE.

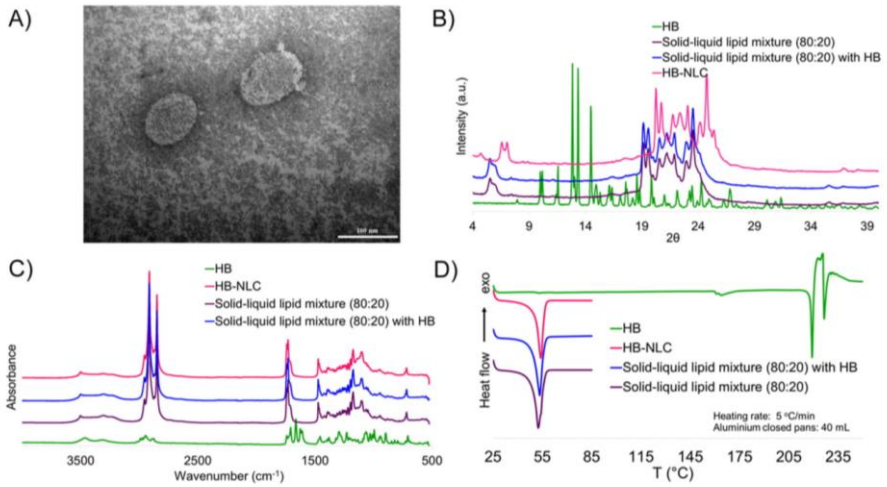


Figure 2. Characterization of optimized HB-NLC and components. **(A)** TEM image (scale bar 100 nm). **(B)** X-ray diffraction patterns. **(C)** FTIR analysis. **(D)** DSC curves.

months while at 25 °C and 37 °C, these parameters change from the first month. ZP is decreasing during time in the three storage conditions (S4). EE is maintained in all cases by over 95%. Best storage temperature is at 4 °C.

Biopharmaceutical behavior

The *in vitro* release of HB-NLC and free-HB shows a first-order release kinetics, with a Fickian's diffusion.[35] Figure 4 A shows a faster release of the drug from the NLC during the first 12 h, after that the speed is decreased. The free-HB has a faster release, achieving a 98% at 24 h while the HB-NLC just released 57%. At 52 h, a 76% release of HB is reached from the NLCs. During this time, the release plateau is not achieved. These results indicate that from the HB-NLC formulation, a prolonged release of HB could be obtained.

The *ex vivo* skin permeation results show that the latency time (Tl) is approximately 40% bigger in HB-NLC in comparison to free-HB, meaning that the HB-NLC system needs 12 h to reach the steady state to begin the permeation of the drug through the skin meanwhile the free-HB just need 7.8 h (Figure 4, *B*). Free-HB shows a faster permeation at all times, reaching six-fold the

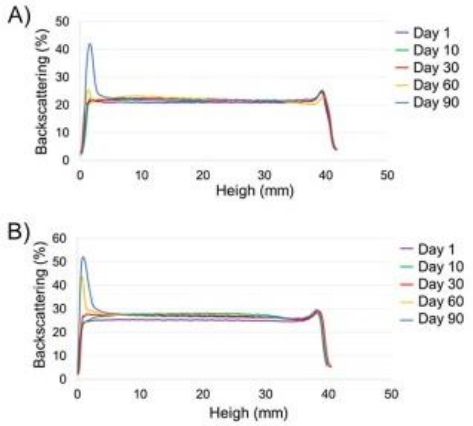
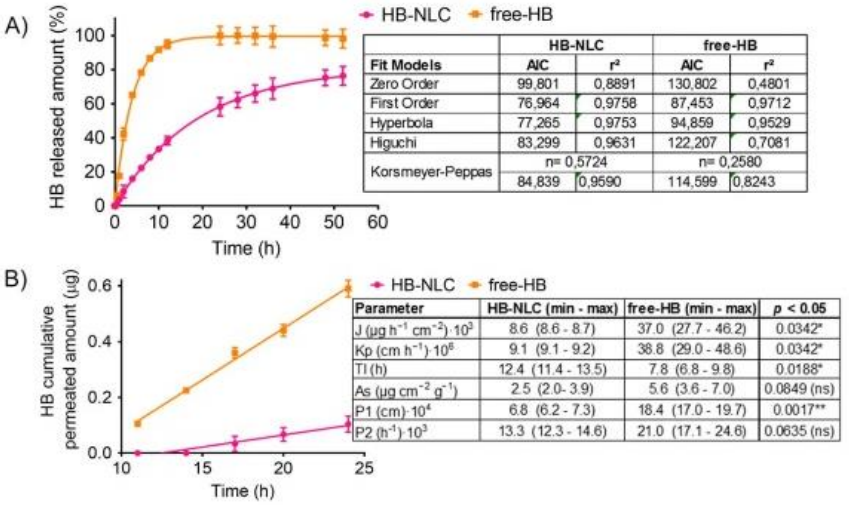


Figure 3. BS profiles of HB-NLC stored at different temperatures. **(A)** 4 °C. **(B)** 25 °C.



	HB-NLC		free-HB	
Fit Models	**AIC**	**r²**	**AIC**	**r²**
Zero Order	99,801	0,8891	130,802	0,4801
First Order	76,964	0,9758	87,453	0,9712
Hyperbola	77,265	0,9753	94,859	0,9529
Higuchi	83,299	0,9631	122,207	0,7081
Korsmeyer-Peppas	n= 0,5724		n= 0,2580	
	84,839	0,9590	114,599	0,8243

Parameter	**HB-NLC (min - max)**	**free-HB (min - max)**	***p* < 0.05**
J (µg h^{-1} cm^{-2})·10^3	8.6 (8.6 - 8.7)	37.0 (27.7 - 46.2)	0.0342*
Kp (cm h^{-1})·10^6	9.1 (9.1 - 9.2)	38.8 (29.0 - 48.6)	0.0342*
Tl (h)	12.4 (11.4 - 13.5)	7.8 (6.8 - 9.8)	0.0188*
As (µg cm^{-2} g^{-1})	2.5 (2.0- 3.9)	5.6 (3.6 - 7.0)	0.0849 (ns)
P1 (cm)·10^4	6.8 (6.2 - 7.3)	18.4 (17.0 - 19.7)	0.0017**
P2 (h^{-1})·10^3	13.3 (12.3 - 14.6)	21.0 (17.1 - 24.6)	0.0635 (ns)

Figure 4. Biopharmaceutical behavior. **(A)** *In vitro* HB release from HB-NLC against free-HB (adjusted to first-order kinetics). **(B)** *Ex vivo* human skin permeation profile of HB from HB- NLC against free-HB and permeation parameters.

amount permeated at 24 h (1.60%). At 24 h, the amount retained and permeated in the skin are 7 and 0.27% respectively considering the initial dose in HB-NLC system. The partition coefficient (P1) NLC-skin, is reduced in HB-NLC compared to free-HB, indicating that a slower release of the drug from the formulation is favored, thus, a slower penetration. The diffusion coefficient (P2), shows a tendency of the medians of HB-NLC to a lower diffusion, lower flow and lower permeability constant, which would indicate that the release of HB to the blood is minor. These results indicate that HB-NLC formulation promotes retention of the drug in the skin over its permeation in comparison with free-HB.

Cytotoxicity and skin tolerance

Cell viability was studied for HaCaT and THP-1 cell cultures. In Figure 5, it is shown that doses less concentrated than $2.5 \cdot 10^{-3}$

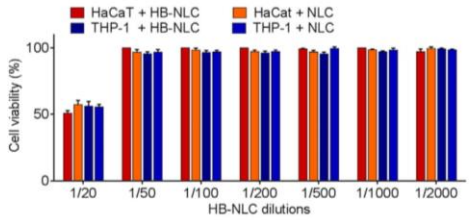


Figure 5. *In vitro* cell viability in THP-1 and HaCaT cell lines. n = 8 cytotoxic independent experiments were performed to reduce variability between the data.

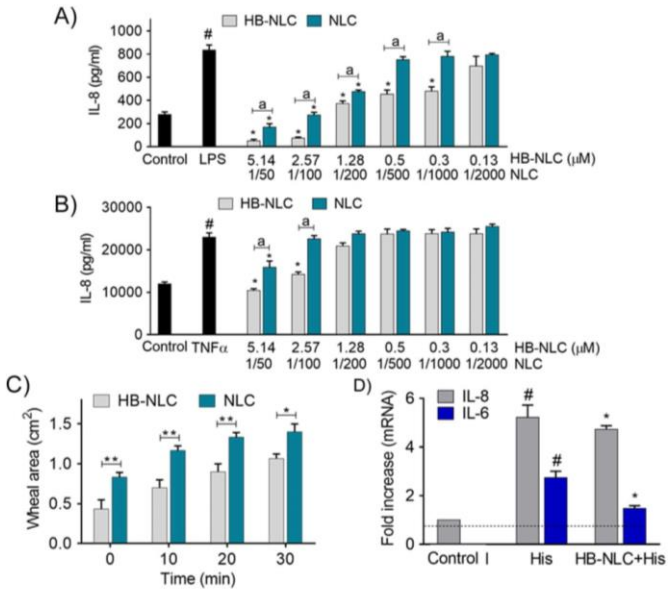


Figure 6. Efficacy results. *In vitro* quantification of IL-8 in cell culture under stimulation (#) and treatment with NLC and HB-NLC. **(A)** THP-1. **(B)** HaCaT. **(C)** *In vivo* Histamine-induced wheal suppression test. **(D)** *Ex vivo* Gene expression of IL-6 and IL-8 from rabbit back samples. (#) comparison with control group ($P \ll 0.05$). (*) comparison with stimulated group. Significant differences * = $P \ll 0.05$; ** = $P \ll 0.01$.

mg/ml of HB, corresponding to dilution 1/50, does not affect cell viability, which is close to 100%. However, to test higher HB-NLC doses, (dilution 1/20) can affect decreasing the viability in both cell lines up to 50%, probably due to the presence of surfactant Tween® 80.[36] The results are similar in presence of HB-NLC and empty-NLC (NLC) indicating that in this form, the presence of HB does not affect cell viability in the studied concentrations.

Skin tolerance results show that after 24 h of exposure to the substance, the values of individual primary irritancy index (Draize test) were "0" for erythema and edema, settling the results obtained in *in vitro* cytotoxicity. HB-NLC is classified as a non-irritant and considered safe for skin administration.[29]

Anti-inflammatory efficacy

In vitro, cell cultures of THP-[1] and HaCat were stimulated with LPS and TNFα respectively, to produce IL-8. In Figure 6, *A* and *B*, both cell cultures, show a significant decrease in the production of IL-8 in the presence of HB-NLC. In THP-1, the decrease in IL-8 is statistically significant up to a concentration of 0.30 μM HB (Dilution 1/1000 HB-NLC). With a 1/50 dilution, there is an evidence that IL-8 decreases up to 75% under control (Figure 6, *A*). In HaCat-1 (Figure 6, *B*) the same result is observed up to 2.57 μM HB (1/100 dilution HB-NLC). *In vitro* results in both cellular types would indicate an anti-inflammatory effect.

In vivo Histamine (His)-induced wheal suppression test results (Figure 6, *C*) show that at all studied times, there are significant differences between the NLC (without drug) and the treatment group. The blisters generated in the HB-NLC group are approximately 35% smaller, indicating an anti-inflammatory effect, confirming *in vitro* efficacy.

Ex vivo results of gene expression are shown in Figure 6, *D*. The stimulation with histamine increases approximately 5-fold the production of IL-8 and almost 3-fold the production of IL-6 against the control. The samples treated with HB-NLC showed a decrease of 52% and 10% of IL-6 and IL-8 respectively in comparison with stimulated histamine without treatment. This result is statistically higher in keratinocytes than monocytes and supports the anti-inflammatory topical effect of the NLC formulation.

Discussion

Considering the chemical characteristics of HB,[37] it is necessary to select the appropriate lipid components and concentration to generate a homogenous matrix capable of solubilizing the drug.[38] Two components were selected to produce the HB-NLC: LAS®, a mixture of caprylic/capric glycerides, with a high solubilizing capacity[39] and Precirol® (HLB = 2), a mixture of esters of palmitic and stearic acids with a melting point between 50 and 60 °C.[40] Having into account the physicochemical properties of both lipids and the melting point of the final mixture, the incorporation of the drug into the lipid matrix is allowed to subsequently generate the HB-NLC in aqueous suspension enhancing the physical stability of the loaded drug.

The independent variables of DoE, were selected to ensure obtaining suitable HB-NLC formulation. It is known that factors such as viscosity can influence NLC properties. In this way, lipid concentrations above 10% generate high viscous systems that increase Z_{av} and size distribution.[41] Due to the above, for these experiments a maximum of 3% of total lipid was established, since higher quantities generated unstable and large particles with high PI. The parameters used for the factorial design, such as the conditions of Ultra-turrax and the high-pressure homogenization conditions[42] were previously determined (S3). Pressure, temperature and number of cycles, directly affect the particle size and the PI resulting in a Z_{av} and PI decrease when higher pressure and more cycles are used.[43] Factorial design showed that all the independent factors studied affected the properties of the HB-NLC for Z_{av}, PI and ZP. This could indicate that there is an "appropriate" ratio between the amount of lipid present and the surfactant capacity of Tween® that can generate the HB-NLC with the optimal characteristics for skin administration. Any different combination of this "proper" ratio, would generate larger particles and a bigger PI.[42,43] The fact that EE only depends on the HB concentration, could be attributed to the high lipophilicity of HB,[37] that generates a strong interaction with the lipid matrix, indicating that a bigger amount of drug directly increases the EE.

TEM characterization, showed that HB-NLC have a round shape, characteristic of this nanosystems type, as the lipids tend to cluster in this (more stable) way in presence of surfactant and water.[44] The interaction studies (X-Ray, FTIR, DSC), showed that the drug and the lipid mixture are compatible, being the drug, dissolved in the lipid matrix without observation of drug crystals, and without peaks that indicate the appearance of new components in the HB-NLC. In all the technics assayed, the HB-NLC profiles were very similar to the lipid mixture's, presenting the characteristic peaks of the major component. These results, would indicate that during manufacturing process of HB-NLC, there is no evidence of the existence of covalent bonds between the drug and the lipid matrix, allowing the HB release from HB-NLC formulation.[45]

The BS and PCS profile confirm that HB-NLC morphometric characteristics (average size and PI) remain constant during 2 months stored at 4 °C. At low temperatures it is ensured that the lipid mixture remains in solid state avoiding melting, possible collisions or aggregation between particles. HB-NLC stability is sensitive to high temperatures (25–37 °C). An increase in the kinetic energy could cause softening and collision of the particles and their possible aggregation increasing the size and PI and generating other physical changes as precipitation, flocculation or creaming in the formulation,[23,46]

Biopharmaceutical behavior determined in the receptor medium of Transcutol/water 70:30, allowed to solubilize the drug and maintain the sink conditions during the experimental time, without affecting the HB-NLC matrix or promoting the penetration of HB through the skin.[20]

First-order release kinetics was selected among the five analyzed models, showing the parameters that fits best to the experimental data, based on a minimum Akaike Information Criterion (AIC) value and a maximum r^2 value.

During the first hours, the faster drug release profile can be explained by drug desorption from the surface of the NLC. After this, a sustained and slower release of the drug can possibly occur by diffusion of HB from the lipid mixture or erosion of the lipid matrix.[26]

According to permeation results, the mechanism of skin penetration would be mainly determined by the vehicle-skin partition coefficient (P1), over the mechanisms involved in the diffusion coefficient (P2). This could be because the high lipophilicity of HB favors the interaction with the NLC matrix delaying its release from the formulation towards the skin. In comparison, the possible skin disruption caused by the surfactant or the occlusive effect of the formulation[38] did not generate a statistical difference in P2 (free-HB v/s HB-NLC). Differences in Tl between free-HB- and HB-NLC confirm P1, indicating a slower permeation of the drug from HB-NLC. This would mean that having a slower drug release and permeation, could generate a system with longer effect with the drug retained in the skin, retarding the passage of HB to the systemic circulation.[25,47]

Commercial formulations (0.05% HB) recommend not to exceed 1.8 mg/day of HB to avoid systemic effects.[48] HB-NLC with a HB concentration of 0.01% has a penetration of 0.27%. These parameters support the safety of the formulation avoiding systemic side effects due to excessive drug permeation.

MTT 24 h results for NLC and HB-NLC showed that from the 1/50 dilution, the cell viability exceeded 90%. It has been described that NLC-corticosteroids could cause cytotoxicity/dose-dependent under certain conditions and that the toxic potential of NLC depends mainly on the concentration and type of surfactant used.[49–51] HB-NLC contains 19 mg/ml of Polysorbate 80, amount considered as

safe for topical use and even for intravenous formulations.[52] Also 48 h were also tested (data not shown) with similar results but longer experimentation times are not recommended because human keratinocytes require fresh medium every 48 h to remove toxic metabolic and to avoid nutrients limitation.[53] The reduction of the cell viability at 1/20 dilution could be due to the presence of Tween® 80 or the lack of nutrients in the growth medium, nevertheless the formulation would be suitable for dermal use.[54]

The results of the *in vivo* Draize skin test (negative for edema, erythema, and irritation) were expected because Precirol®, LAS® (major components of the formulation) and Tween®80 (surfactant) are biocompatible, biodegradable and with low skin toxicity.[39,55]

The *in vitro* anti-inflammatory efficacy of HB-NLC, determined by the quantification of pro-inflammatory cytokine IL-8 produced in HaCaT and THP-1 cell cultures[56–58] showed a reduction of this inflammatory marker in both cases. This would suggest that the drug is able to leave the nanoparticle to exert its intracellular action, typical mechanisms of corticosteroids.[59] These results have been compared with those obtained by *in vivo* histamine-induced wheal suppression test, a simple and non-invasive method that allows emulating the inflammatory conditions (redness and temperature increase) of skin diseases when a corticoid would be commonly used.[30] It has been described, that depending on the concentration, NLC can generate an occlusive effect on the skin.[11] Even if there were an occlusive effect with HB-NLC, given the low amount of lipid present in the formulation, this effect would not imply a significant increase in the permeation of HB.

The anti-inflammatory effect of HB-NLC is evidenced by the occurrence of a smaller blister compared to the control area at all the studied times. Along with that, tissue analysis by RT-qPCR shows that the production of mRNA for IL-6 and IL-8 decreases under treatment with HB-NLC, confirming the *in vivo* results.

In vivo and *in vitro* efficacy test agree and demonstrate the anti-inflammatory effect of the HB-NLC formulation.

In summary, a new HB-NLC formulation with suitable characteristics for skin administration was developed. The formulation is stable during a period about two months at 4 °C, after this, shaking the formula would be recommended before use to remove the possible sediment in the bottom of the vial. The release of the drug from the NLC occurs in a sustained manner and its retention in the skin is promoted over the penetration at the systemic level increasing the safety of use for prolonged treatments. *In vivo, in vitro and ex vivo* effectiveness evaluated, indicate an anti-inflammatory activity. Based on the acquired results, we consider that the HB-NLC formulation is an adequate alternative for the treatment of inflammatory skin diseases.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2019.102026.

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3.3. Nanostructured lipid carriers loaded with Halobetasol propionate for topical treatment of inflammation: Development, characterization, biopharmaceutical behavior and therapeutic efficacy of gel dosage forms.

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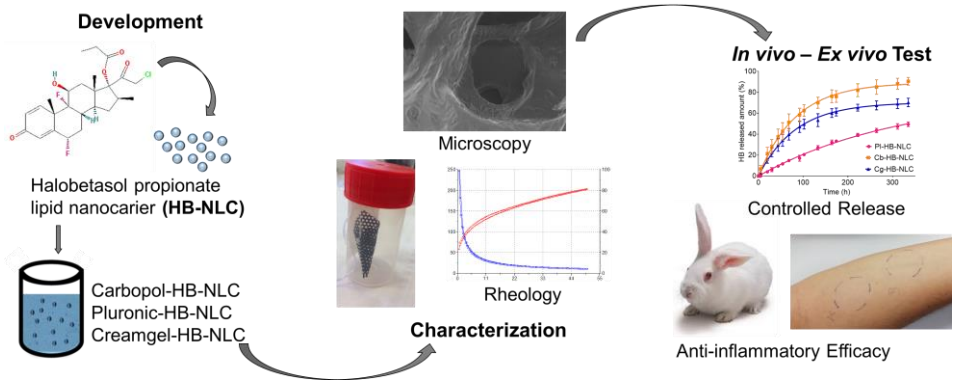

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Nanostructured lipid carriers loaded with Halobetasol propionate for topical treatment of inflammation: Development, characterization, biopharmaceutical behavior and therapeutic efficacy of gel dosage forms

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ABSTRACT

The aim of this research was the development and characterization of three gel dosage forms of Halobetasol propionate loaded lipid nanoparticles (HB-NLC) for the treatment of inflammatory skin diseases. A Pluronic gel (Pl-HB-NLC), a Carbopol gel (Cb-HB-NLC) and a Cremigel (Cg-HB-NLC), were characterized for stability, swelling, degradation, porosity and rheology. The biopharmaceutical behavior of *in vitro* release and *ex vivo* permeation, along with microbiological stability were also evaluated. Tolerance and therapeutic efficacy were determined *in vivo*. The gels proved to have eudermic pH and to be effective to improve HB-NLC stability for more than 6 months. *In vitro* drug release profiles were adjusted to a first order (Pl-HB-NLC, Cg-HB-NLC) and hyperbola (Cb-HB-NLC) kinetic models, revealing sustained drug release. *Ex vivo* biopharmaceutical behavior showed slow drug penetration through skin, delaying the drug entrance into systemic circulation. The formulations were effective in reducing inflammation with a lower drug dose in comparison with existing treatments, obtaining the fastest effect when using Pl-HB-NLC. After application of the formulations in volunteers, no irritation, redness or edema reactions were detected, plus, an enhancement of the biomechanical properties of the skin was evidenciated. Therefore, the results indicate that these formulations are a suitable alternative to current treatments.

1. Introduction

The skin is the largest organ in the body and its principal function is to separate the external from the internal environment. In the face of external (and sometimes internal) stimuli or aggressions, the skin responds in a non-specific and innate way, triggering the inflammatory process (Pasparakis et al., 2014). This process is generally characterized by the presence of edema, increased temperature, itching and redness of the area. Diseases such as psoriasis, allergies, atopic dermatitis and eczema usually produce this type of symptomatology chronically (Boguniewicz and Leung, 2011; Eyerich and Novak, 2013). Due to their great anti-inflammatory action, corticosteroid are drugs of choice for a wide variety of skin diseases (Al-Dabagh et al., 2014). They have several mechanisms of action which allow them to produce local and systemic effects (Ramamoorthy and Cidlowski, 2016). Derived from its multiple mechanisms of action, together with the desired treatment, undesirable local effects such as pigmentation and skin thinning or systemic effects such as metabolic alterations like Cushing's syndrome

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can be produced (Dhar et al., 2014). To improve their characteristics, whether controlling their potency, increasing their desired effects or reducing side effects, different semisolid dosage forms for topical administration have been developed. Creams, gels and lotions have been used for the treatment of diverse skin diseases (Herz et al., 1991; Mancuso et al., 2003; Lowe et al., 2005). Along with this, there are other more technological methods of dosage forms such as the first and second generation of lipid nanoparticles, known as solid lipid nanoparticles (SLN) and nanostructured lipid carrier (NLC), respectively (Müller et al., 2000, 2007). Lipid nanoparticles have been extensively studied to control the penetration and effects of corticosteroids generally considered "high potency", because they are the most likely to cause severe adverse effects, reason why, traditional treatments are recommended for short periods of time (two weeks) despite many times, treating diseases with chronic symptoms (Coureau et al., 2008; Jensen et al., 2010; Bikkad et al., 2014; Carvajal-Vidal et al., 2019).

With the aim to allow prolonged treatments reducing potential systemic side effects, improve skin characteristics and promote the local effect of a "super potent" corticosteroid, over its systemic action, three topical gel dosage forms based on Pluronic (Pl-HB-NLC), Carbopol (Cb-HB-NLC) and Sepigel® (Cg-HB-NLC), containing Halobetasol propionate (HB) loaded nanostructured lipid carriers (NLC), with a 20% of the regular dose have been developed to obtain a new controlled release system of HB as an alternative to current topical treatments.

2. Materials and methods

2.1. Materials

HB was obtained from Capot Chemical Company Limited (Hangzhou, China), LAS (PEG-8 caprylic/capric glycerides) and Precirol ATO® 5 (Glyceryl distearate) were kindly gifted from Gattefossé (Madrid, Spain). Tween® 80 (Polysorbate 80), Isopropyl myristate and Glycerin, were purchased to Sigma Aldrich (Madrid, Spain). Sepigel® 305 (Sep) was bought from Seppic (Paris, France). Polymers Carbopol® 940 (Cb) and Pluronic® F-127 (Pl) were obtained from Fagron Iberica (Terrassa, Spain). All others chemical reagents and components used in this research were of analytical grade. A Millipore Milli-Q Plus system was used to obtain purified water.

2.2. Gel elaboration

HB-NLC suspension was previously produced by high-pressure homogenization method (Homogeniser FPG 12800, Stansted, United Kingdom) (Neupane et al., 2014) with Tween® 80 as surfactant and LAS and Precirol ATO 5® as liquid lipid and solid lipid respectively, as described elsewhere (Carvajal-Vidal et al., 2019).

Three gels were prepared at room temperature with the HB-NLC suspension used instead of pure water. Cg-HB-NLC was prepared by driving the aqueous phase (HB-NLC and glycerin 10%) over the oil phase (Sep 3%, isopropyl myristate 3%) under constant magnetic agitation until mixture homogenization. Cb-HB-NLC was made with 2% (w/v) of polymer and 1% (w/v) of glycerin dissolved directly in HB-NLC suspension under magnetic stirring for 24 h. After, the formulation was stabilized with triethanolamine to ensure a eudermic pH. Pl-HB-NLC was made gradually dissolving the polymer (20%) into the HB-NLC suspension under continuous agitation until there was no residual powder. All formulations after preparation were stored at 4 and 25 °C until following studies.

2.3. Macroscopic and microscopic determinations

The gels were macroscopically studied for color, viscosity and odor immediately and six months after being prepared, to detect visual changes or any instability signs such as phase separation, precipitation or creaming. Fresh formulations were microscopically analyzed to evaluate their droplet size and homogeneity degree using an optical Leica DM 1000 LED light microscope (Leica Microsystems, Wetzlar, Germany) with a camera Leica EC3 (Leica Microsystems, Wetzlar, Germany) at 40x magnifying power, images were taken directly from the formulations without dilution. Meanwhile, the pH was measured with a CRISON micro-pH 200 microprocessor controlled pH-meter (Crison Instruments S.A., Barcelona, Spain) at room temperature (25 °C).

Additionally, the structure of the formulations was studied by Scanning Electron Microscopy (SEM) with a J-7001F (JEOL Inc., MA, USA) with a secondary electron detector (Everhart-Thornley type). The sample was deposited directly on the microscope sample holder, and the subsequent observation was carried out, without the need to coat them, under high vacuum and low voltage (1 kV) conditions (Barshack et al., 2004).

2.4. Rheological behavior and extensibility

Rheological measurements were determined for each formulation using a rotational Haake RheosStress RS1 rheometer (Thermo Fisher Scientific, Karlsruhe, Germany) prepared with a cone-plate geometry (Haake C60-2° Ti, 60 mm diameter, 0.106 mm gap between plates) connected to a temperature control devise at 25 °C (Thermo Haake Phoenix II + Haake C25P). The data were recorded with samples under a program consisting of 3 steps. First, a period of ramp-up from 0 to 50 s^{-1} for 3 min. Second, a constant shear rate period at 50 s^{-1} during 1 min. Third, a ramp- down period from 50 s^{-1} to 0 during 3 min. From the constant shear stretch at 50 s^{-1}, the steady-state viscosity was also determined. Pl-HB-NLC sol-gel transition temperature was determined placing the gel in a water bath under continuous stirring with a magnetic bar. The temperature of the gel was gradually increased from 20 to 38 °C. It was considered that the gelation temperature was reached when the magnetic bar stopped (Brugués et al., 2015). The experiment was accomplished by triplicate.

The extensibility index was determined at room temperature by setting 25 mg of gel between two millimeter microscope slides. The samples were compressed by placing increasing weights (2, 5, 10, 20, 50 and 100 g) on it for 30 s. The diameter (mm) of the formed circle by the gel was noted. For each weight, a diameter of gel was obtained with which the increase in surface area (mm^2) of the formulation was calculated as a function of the increasing weights applied (Campaña-Seoane et al., 2014). The experiment was accomplished by triplicate.

2.5. Swelling, degradation and porosity index

The swelling ratio (SR), the degradation tests and the porosity index (P) were evaluated by a gravimetric method. To evaluate the SR of each formulation, a specified weight of dried gels were immersed in PBS solution (pH = 5.5 at 32 °C) for 1.5 h. for Cb-HB-NLC, 24 min. for Cg-HB-NLC and 4 min. for Pl-HB-NLC. At defined times, previously determined, the samples were taken out from the solution and the weight gain due to PBS uptake was measured. The test was performed in triplicate and the SR% was calculated based on the following equation:

$$SR\% = \frac{(Ws - Wd)}{Wd} \cdot 100 \quad (1)$$

where Ws represent the weight of swollen gel at different times and Wd is the initial dried gel weight (Zhao et al., 2005; Lee and Bucknall, 2008).

Degradation test was calculated as percentage of weight loss (WL) by immersing weighted amounts of fresh gel in a PBS solution (pH = 5.5 at 32 °C) during 6.5 h. for Cb-HB-NLC, 1 h. for Cg-HB-NLC and 80 min. for Pl-HB-NLC. At regular time intervals, the samples were withdrawal, the excess of water was removed and the weight was measured. WL was calculated using the equation:

$$WL(\%) = \frac{(Wi - Wd)}{Wi} \bullet 100 \quad (2)$$

where, Wi is the initial fresh gel weight and Wd is the gel weight at different time intervals (Mallandrich et al., 2017).

The P was measured by solvent replaced method. A total of 0.5 g of the dried gel was submerged in absolute ethanol. During different periods of time, the sample was weighted until constant weight. P was calculated based on the following equation:

$$P = \left(\frac{W2 - W1}{\rho \times V}\right) \bullet 100 \quad (3)$$

where, W_1 is the weight of the dried gel. W_2 is the final weigh of the gel (with ethanol), ρ is the density of absolute ethanol and V is the volume of applied gel to the test.

2.6. Stability test

Cg-HB-NLC, Cb-HB-NLC and Pl-HB-NLC were stored at 4 and 25 °C for 6 months. During this time, the physical stability of the formulations needed to be assessed. For this purpose, stability studies were carried out through the analysis of light backscattering (BS) by using a Turbiscan®Lab equipment to identify possible destabilization phenomena such as sedimentation or creaming (among others). A sample of 10 g of each gel was placed in a glass measuring cell. The radiation source used was a pulsed near infrared light-emitting diode LED (λ = 880 nm) read by a backscattering detector located at an angle of 45° from the incident beam. Measurements (one scan every five minutes during 15 min) were taken 1 day after preparation of the formulation and at 1, 2 and 6 months.

2.7. Microbiological stability

6 months after being prepared, the microbiological load in the gels was measured. The counting of viable mesophilic microorganisms was carried out through inoculation by inclusion in plates of TSA (Triptona Soy Agar, OXOID Ref. CM131B) and Sabouraud Dextrose Agar supplemented with chloramphenicol (OXOID Ref. CM CM0041) for fungi and yeasts. The sample was incubated at 30 ± 2 °C for 5 days. To verify the absence of *E. coli*, *Staphylococcus aureus* and *Candida albicans*, 1 mL of each sample was inoculated in TSB broth tubes (Triptona Soy Caldo de OXOID, Ref. CM0129B) and incubated at 35 ± 2 °C for 72 h. After that, striae by exhaustion were made in selective media for the microorganisms previously described (Ratajczak et al. 2015).

2.8. Biopharmaceutical behavior

2.8.1. In vitro release

To determine and compare the *in vitro* HB release of each gel, a study was carried out in Franz-type diffusion cells (FDC 400, Crown Glass, Somerville, NY, USA) (Gonzalez-Pizarro et al., 2019). A cellulose dialysis membrane, MW 12000–14000 Da (Iberoamerica, Spain) was selected and after hydration was placed between the donor and the receptor compartment with a diffusion area of 2.54 cm^2. For hydrating the membrane and as a receptor medium a solution of Transcutol®/water (70:30) was used. When starting the experiment, in the donor compartment, 300 mg of each gel were added in complete contact with the dialysis membrane. At determined times, samples of 300 µl were collected from the receptor compartment with a syringe and replaced with new receptor medium. The test was carried out during 335 h. assuring sink conditions and a constant temperature of 32 ± 0.5 °C. The HB content in the samples was analyzed by RP-HPLC by a methodology described elsewhere (Carvajal-Vidal et al., 2017). The release test was performed by triplicate and the cumulative amount of HB, Akaike's information criterion (AIC) and correlation coefficient (r^2) were calculated to determine the best fitting release model (Ramos et al., 2016) and to compare release characteristics between formulations.

Kinetic equations used to fit the amount of HB released:

$$\text{Zero order: } \frac{Qt}{Q\infty}(\%) = k \bullet t \quad (4)$$

$$\text{First order: } \frac{Qt}{Q\infty}(\%) = 1 - e^{-kt} \quad (5)$$

$$\text{Hyperbola: } \frac{dQ}{dt} = \frac{Vm \bullet Q}{Km + Q} \quad (6)$$

$$\text{Higuchi: } \frac{Qt}{Q\infty}(\%) = kh \bullet t^{1/2} \quad (7)$$

$$\text{Korsmeyer} - \text{Peppas: } \frac{Qt}{Q\infty}(\%) = k \bullet t^{n} \quad (8)$$

where Q_t corresponds to the amount of drug released at time (t), $Q\infty$ corresponds to the maximum amount of drug released, "k" is the release rate constant (concentration/t), and "n" is the diffusion release indicative of the drug release mechanism. (Costa & Sousa Lobo, 2001). Vm represents the maximum process speed and Km corresponds to the amount of drug relative to a speed that is half of the maximum speed (Berrozpe et al., 2008).

2.8.2. Ex vivo skin permeation

For each formulation, a permeation test with human skin was performed in Franz-type cells (FDC 400, Crown Glass, Somerville, NY, USA) (Gonzalez-Pizarro et al., 2019) for a period of 36 h. The human skin was voluntary donated under informed consent by a female patient undergoing an abdominal plastic surgery. The procedure and the experimental protocol were approved by the Bioethics Committee of the Barcelona-SCIAS Hospital (Barcelona, Spain). Once obtained, the skin was frozen at −20 °C to facilitate its manipulation. Subsequently, based on international guidelines (Organization for Economic Co-Operation and Development; European Centre for the Validation of Alternative Methods), 0.4 mm thick pieces of skin cut with a dermatome (Model GA 630, Aesculap, Tuttlingen, Germany) were used as a permeation membrane. The skin was placed in the Franz cells between the donor compartment and the receptor compartment, with the stratum corneum facing the upper chamber and with a diffusion area of 0.64 cm^2. Once assembled, the skin barrier integrity was evaluated by measuring transepidermal water loss (TEWL) (TEWL-meter TM210, Courage & Khazaka, Koln, Germany) with the probe placed in the donor compartment very close to the skin. Human skin pieces exhibiting TEWL values below 10 g/(m^2·h) were used for the experiment (Mallandrich et al., 2017). The temperature of the system was kept constant at 32 ± 2 °C by a thermoregulated water bath during the complete experiment, and a solution of Transcutol/water (70:30) was used as a receptor medium in the receptor compartment. A total of 300 mg of each formulation were placed on the donor compartment in direct contact with the skin membrane. At certain times, an aliquot of 300 µl from the receptor chamber was withdrawn and replaced with fresh medium solution until 36 h. of contact. The quantity of permeated HB was determined by RP-HPLC. Later, permeation parameters such as flux (J), permeability coefficient (Kp), permeated amount at 36 h (A_{36}), were calculated using a linear least-squares regression model. The samples were performed by triplicate and evaluated in skin from the same donor to avoid variations in the response due to biological differences of the skin, and only detect differences due to characteristics of each formulation.

2.9. In vivo and ex vivo assays

2.9.1. Animals

Following the existing international guidelines (Organization for

Economic Co-Operation and Development (OECD), 2019), New Zealand male albino rabbits of 2.0–2.2 kg weight were used for the *in vivo* experimentation. Rabbits were kept in standard cages with food and water *ad libitum* and under controlled ambient conditions (25 ± 1 °C, 50–60% relative humidity). According with the Catalonian government regulations, the experimental protocols were approved by the Animal Research Ethical Committee of the University of Barcelona.

2.9.2. In vivo and ex vivo skin tolerance

Two squares of 5 × 5 cm^2 (left and right) were shaved with an electric razor on the back of the rabbit. One section was used as a control and the other was scraped with a lancet. 24 h later, 0.5 mL of the formulation (each separately) were applied to the shaved segments and left uncovered. After 24 h of contact between the formulations and rabbit skin, the excess was removed and the skin was macroscopic evaluated for edema and erythema according to a graduated scale of 0 to 4 in both cases. The individual primary irritancy index was determined for each rabbit based on the scale originally described by Draize (Draize et al., 1944; Zuang et al., 2005). The results allow to determine if a substance is "non-irritant", "mildly irritant", "moderately irritant", or "severely irritant". The test was performed by triplicate.

2.9.3. Histological studies

After macroscopic evaluation and to corroborate the results of the Daize test, histological evaluation of the tissue in contact for 24 h with the formulations was performed by optical microscopy. The samples were obtained and fixed in 4% formaldehyde, included in paraffin and stained using the eosin/hematoxylin technique (Jörundsson et al., 1999).

2.9.4. In vivo efficacy

The anti-inflammatory efficacy of each gel was individually evaluated using the histamine-induced wheal suppression test, in order to compare the kinetic parameters between them (Abidi et al., 2010). The procedure consists of shaving the back of the rabbit to leave an area clear of hair that allows direct visualization of the skin. Subsequently, a control site and a treatment site were determined. A total of 0.5 g of gel were applied in the treatment area, and 0.5 g of gel without HB were applied in the control area, for equality of conditions in both zones. After 1 h, with a cotton swab, any superficial excess of formulation was delicately cleaned and the test was performed. A total amount of 0.05 mL of histamine dihydrochloride solution (0.1%) were intradermal injected with an insulin syringe in both sites. 10, 20 and 30 min. after the injection, the size of the blebs generated were measured (cm^2) with a caliper and compared, treatment zone versus control zone. Considering the approved protocol and the 3Rs principles (Passantino, 2008) the test was performed by triplicate.

2.10. Skin integrity parameters (in vivo tolerance in humans)

Based on the recommendation of the Declaration of Helsinki (General Assembly of the World Medical Association, 2014), the experimental protocol for this assay was approved by the Ethics Committee of the University of Barcelona (IRB12300003099). 20 volunteers with healthy skin, 10 men and 10 women, aged between 25 and 55 years old, gave their informed and written consent for their participation in the study. Two days before measurements and during the study, volunteers were instructed to avoid using skin care products or skin lotions on the area to be tested. On the day of the measurement, volunteers were asked to stand in the test room for at least 30 min. to stabilize at ambient conditions (25 °C and 50–60% humidity). Biomechanical properties measurements were made on the inside of the forearm, before and 2 h after that the formulation was uniformly applied (0.1 g/cm^2). Skin temperature was measure with a Skin Thermometer® ST500 (Courage-Khazaka electronic GmbH, Cologne, Germany) as a general control parameter. The TEWL was measured with a Tewameter® TM 300 (Courage-Khazaka electronic GmbH, Cologne, Germany) to determine the integrity of the skin barrier function and hydration of the stratum corneum was measured using a Corneometer ® CM 825 (Courage-Khazaka electronic GmbH, Cologne, Germany).

2.11. Statistical analysis

The statistical analysis and data comparisons were achieve using two tailed *t*-test with a significance of $\alpha < 0.05$. To confirm the normality of the data and the equality of the variances, F test was accomplished. In cases where the data had abnormal distribution, Wilcoxon paired test was performed with a significance of $\alpha < 0.05$. For the data treatment GraphPad Prism® 6.01 software (GraphPad Software Inc., San Diego, CA, USA) was used and the results are presented as mean value ± standard deviation (SD).

3. Results and discussion

3.1. Gels characterization

The three gels formed containing HB-NLC, presented characteristic odor due to the components and opaque white color probably due to the suspension of HB-NLC used instead of water. Cg-HB-NLC and Cb-HB-NLC showed high viscosity and low fluidity when inverting the container tube. Pl-HB-NLC was liquid at room temperature, and gelled at skin temperature (32 ± 0.5 °C). The pH, determined at 25 ± 1 °C, was maintained in the eudermic range between 5.2 and 6.5 (Jiménez et al., 2002) just after preparation and until 6 months of storage at room temperature (25 ± 2 °C) and 4 ± 2 °C.

The results of optical microscopy (Fig. 1) show that Cg-HB-NLC is a monodisperse emulsion with droplets between 4 and 5 μm, the dark spots that are seen, have a size smaller than 1 μm and could be HB-NLC accumulations. The gels Cb-HB-NLC and Pl-HB-NLC present a thin and homogeneous framework with particles smaller than 500 nm.

On the other hand, SEM results show for Pl-HB-NLC a moderately lax matrix generated in the aqueous HB-NLC medium by the crosslinking of the polymer fibers. These spaces of approximately 600 μm size would allow the entry of water and the subsequent mobilization of nanoparticles (Zav ~ 200 nm) into the gel matrix, promoting their release from the formulation.

3.2. Rheological studies and extensibility test

The rheological results (Fig. 2) revealed that Cg-HB-NLC and Pl-HB-NLC had a pseudoplastic behavior in the ascendant and descendant stretch, having Cross equation as the best fitting model in both cases ($r^2 > 0.9998$). Thixotropic behavior was not detected in these two formulations. The viscosity at 50 s^{-1} was 4053.11 ± 9.10 mPa·s and 325.42 ± 1.61 mPa·s, respectively. Meanwhile, Cb-HB-NLC presented a very high initial viscoelastic behavior (critic shear 8 s^{-1}) and due to thicker crisscross of the system, the viscosity could not be determined at 50 s^{-1}. However, the viscosity comparison between gels could be performed in the ascending sections for a shear rate value of 5 s^{-1}. Being respectively higher to lower viscosity Cb-HB-NLC (167.70 ± 5.03 Pa·s) > Cg-HB-NLC (22.35 ± 0.40 Pa·s) > Pl-HB-NLC (0.60 ± 0.01 Pa·s). The rigidity of the polymer network, what determines the viscosity of the formulation, induces the resistance to flow, therefore, more rigid and compact framework presents greater viscosity, as can be seen in Cb-HB-NLC (Fig. 2c), while in more fluid formulations, it assume that crosslinking is more lax as seen in the case of Pl-HB-NLC (Fig. 2b). Before being incorporated into the gels, HB-NLC presented a Newtonian behavior and a viscosity of 1.75 ± 0.02 mPa·s regardless of the shear rate. An increase in viscosity due to the addition of gelling components could favor the stability of the HB-NLC system (Tohver et al., 2001). Repeatability was very good for Cg-HB-NL, Pl-HB-NLC and HB-NLC suspension exhibiting variations < 2%.

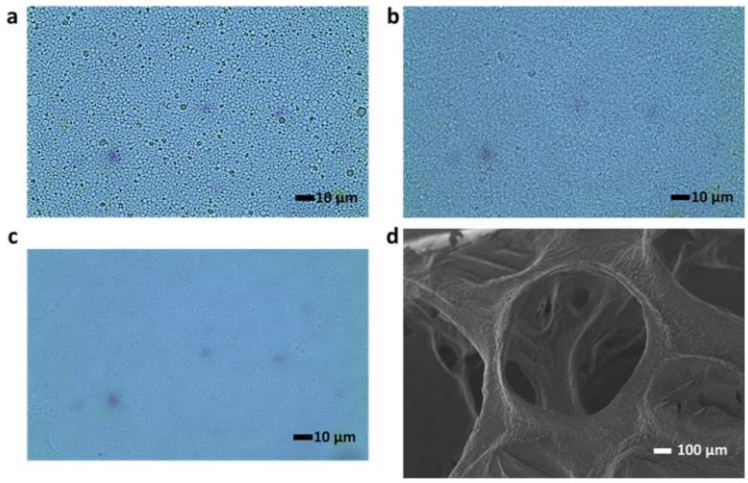


Fig. 1. Images by optical microscopy of (a) Cg-HB-NLC, (b) Cb-HB-NLC, (c) Pl-HB-NLC and (d) SEM image of Pl-HB-NLC.

The extensibility results performed at 25 ± 2 °C are shown in Fig. 3. The linear zone of the graph where the extensibility response is proportional to the weight used, is between 20 and 100 g. It can be seen that at this temperature and 50 g compression the order of decreasing extensibility is Pl-HB-NLC (3276 ± 15 mm^2) > Cg-HB-NLC (771 ± 14 mm^2) > Cb-HB-NLC (328 ± 17 mm^2). Inverse classification to that obtained with viscosity values. This is logical given that the more fluid the system, the more extensible is (Suñer-Carbó et al., 2017). In this sense, Pl-HB-NLC is the most extensible and less viscous formulation, contrary to Cb-HB-NLC, where a strong elastic behavior is

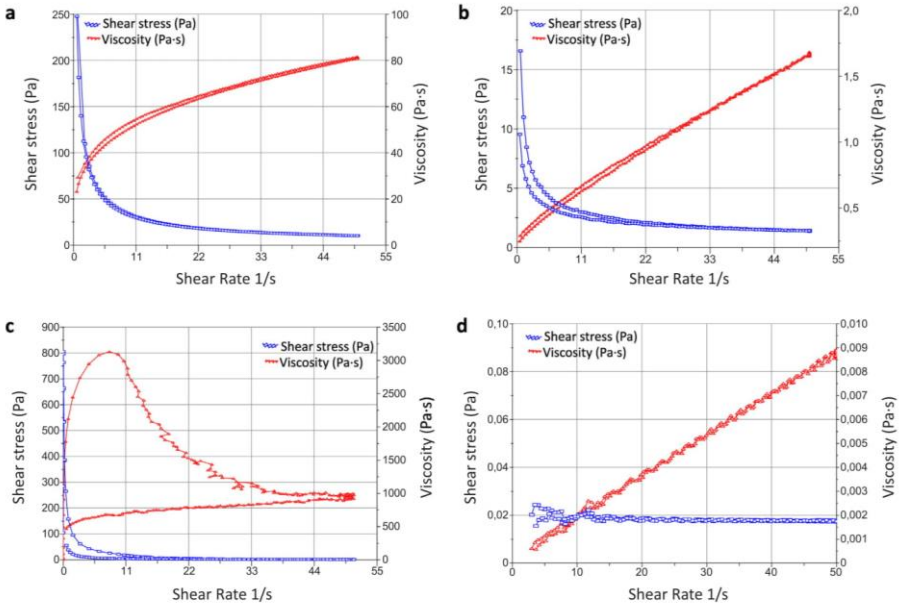


Fig. 2. Rheograms of the tested formulations measured at 25 °C. (a) Cg-HB-NLC, (b) Pl-HB-NLC, (c) Cb-HB-NLC, (d) HB-NLC suspension.

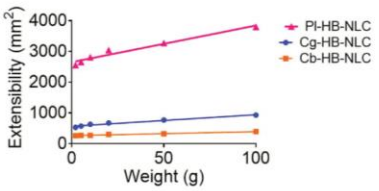


Fig. 3. Extensibility values measured at 25 °C of the Cb-HB-NLC, Pl-HB-NLC and Cg-HB-NLC formulations. Results represented as mean value ± SD.

evidenced, making this gel behave more like a solid than a liquid.

3.3. Swelling, degradation test and porosity index

The swelling process of the three gels, Pl-HB-NLC, Cb-HB-NLC and Cg-HB-NLC, follow a first order kinetics (Fickian kinetic) (Fig. 4) represented by the kinetic constant (k) 1.48 min^{-1}, 0.05 min^{-1} and 0.14 min^{-1}, respectively. The swelling kinetics can be affected by the pH, temperature and the ratio and concentration of the gel cross-linking (Abdul and Rajab, 2014). The results show that Pl-HB-NLC is completely solubilized in 4 min reaching a Q_{max} = 1.75%. Meanwhile, Cb-HB-NLC has a Q_{max} = 7.39% at 90 min and Cg-HB-NLC reaches a Q_{max} = 1.5% after 24 min. It has been described that the rate and amount of PBS captured are related to the amount of functional groups of the gelling structures, influencing the mobility of the polymer chain, thereby decreasing or increasing the SR (Samuel and Bhise, 2017). In this case, the gels have similar pH (~5), same HB-NLC water base concentration and they were treated under the same temperature which would lead us to consider that the differences are due solely to the matrix intercrossing. Cb-HB-NLC has a high cross-linking level with low flexibility (Tang et al., 2005) this would make it a more compact gel, endorsing the slower but higher PBS uptake found in comparison to Pl-HB-NLC and Cg-HB-NLC which show lower level of crosslinking.

The degradation process for the studied gels showed first order kinetics (Fig. 4). This means that the weight loss depends on the concentration of polymer (gel) and therefore, that at higher concentration, greater degradation velocity, decreasing the speed of the process over time. For Pl-HB-NLC the process occurs in 80 min. with a k = 0.04 min^{-1}. Whereas, for Cb-HB-NLC it occurs in 6.5 h with a k = 0.25 h^{-1} and for Cg-HB-NLC in 60 min with a k = 0.12 min^{-1}. The differences between the gels could be due to the level of cross-linking of the polymer, for Cb-HB-NLC, the polymer has a high level of cross-linking with low flexibility (Tang et al., 2005) which could influence reducing the speed of the process. While for laxer cross-linking formulations such as Pl-HB-NLC, the process would be favored (Mallandrich et al., 2017).

The results of P in decreasing order were 5.82 ± 1.04% for Pl-HB-NLC > 0.89 ± 0.12% for Cg-HB-NLC and 0.48 ± 0.07% for Cb-HB-NLC. A decreased P could occur due to an increase in the concentration of the cross-linking agent, increasing the association between the monomer and the polymer (Bukhari et al., 2015). On the other hand, gels with a small pore size have a strong viscous coupling between the formed network and the solvent, suppressing the movement of the polymer network in relation to the solvent (de Cagny et al., 2016). These P results are in agreement with the viscosity values where Cb-HB-NLC has lower P and high viscosity, contrary to Pl-HB-NLC, where a higher P correlates with a lower viscosity.

3.4. Stability test

The physical stability of the samples stored at 4 and 25 °C revealed that the gels are highly stable for more than 6 months at both temperatures (Fig. 5). However in comparison, Cg-HB-NLC showed slightly changes at 25 °C (Fig. 5d), probably because its composition is more complex than Pl-HB-NLC and Cb-HB-NLC. The temperature would alter the interaction between the lipid and polymer components, promoting cross-linking alteration and therefore promoting the instability of the sample (Limón et al., 2015; Ji et al., 2015). Considering the obtained results, even if both storage temperatures are adequate, the temperature at which the formulations remain more stable is at 4 °C, which is consistent with the temperature storage for HB-NLC suspension. Before incorporation into the gels, HB-NLC proved to be stable by itself for one month (Carvajal-Vidal et al., 2019). So, the incorporation of the HB-NLC into the gel matrix, improves their storage stability 6 times. These results are in accordance and could be explained by viscosity changes. A

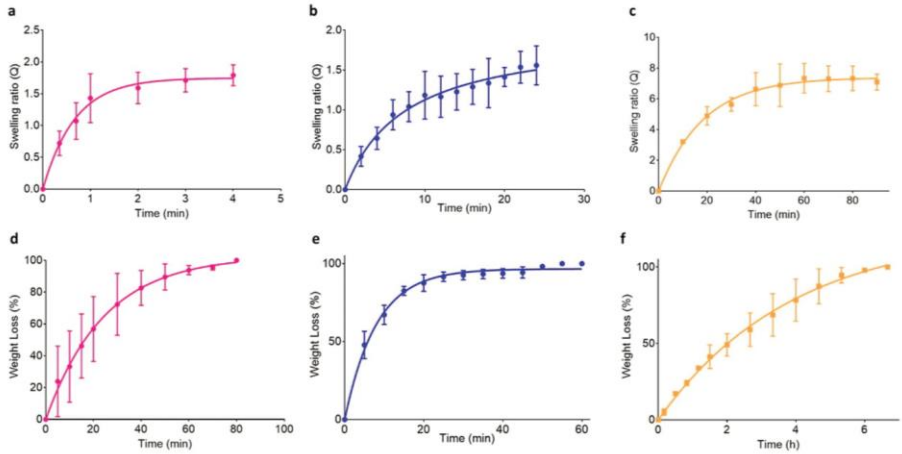


Fig. 4. Swelling kinetics of (a) Pl-HB-NLC, (b) Cg-HB-NLC, (c) Cb-HB-NLC and WL% of (d) Pl-HB-NLC, (e) Cg-HB-NLC, (f) Cb-HB-NLC. Results represented as mean ± SD.

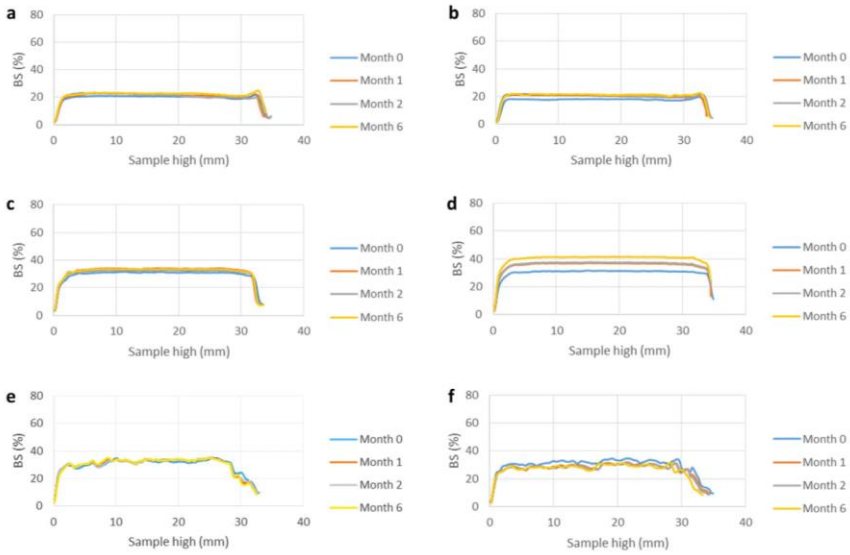


Fig. 5. Backscattering gels profiles of Pl-HB-NLC at 4 °C (a) and 25 °C (b), Cg-HB-NLC at 4 °C (c) and 25 °C (d), Cb-HB-NLC at 4 °C (e) and 25 °C (f).

more viscous matrix would decrease the free movement of the particles, and their aggregation or sedimentation could be delayed or even avoided compared to the original aqueous system.

3.5. Microbiological assay

All evaluated products showed the same behavior at microbiological level, those stored at room temperature and at 4 °C. Regarding the total mesophilic, fungal and yeast count, we can conclude that the bacterial load is less than 10 cfu/g in all samples. Seeding in the corresponding selective media from the enrichments in TSB broth, allowed to verify the absence of pathogenic microorganisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans.* The gels were formulated without a preservative medium, therefore the absence of microbiological growth, could mean that the amount of free water in the formulations is not enough to promote the proliferation of microorganisms or that the components of the nanoparticles (despite being lipid) do not favor the microbiological multiplication.

3.6. Biopharmaceutical behavior

3.6.1. In vitro release

The *in vitro* release profile, determined by Franz cells are shown in Fig. 6a. As can be seen, during the first h. there is a rapid release of HB, reaching a 62.5% from Cb-HB-NLC at 100 h, which continues increasing until it becomes sustained without achieving the plateau during the study period. The same happens with Pl-HB-NLC, the plateau is not evident, but it can be seen that the release process is much slower, reaching only a 49.8% of HB released in 300 h. This behavior could be due to the lipid characteristics of the drug, because its solubility in the polymer matrix is disadvantaged in contrast to NLC matrix, thus delaying the diffusion of HB from the NLC (Roseman, 1972). In comparison, Cg-HB-NLC seems to release a maximum of 65.3% of the total formulation content at 220 h. Pl-HB-NLC and Cg-HB-NLC follows a first order kinetics, with an initially rapid and subsequently sustained release over time, characteristic of SLN (Venkateswarlu and Manjunath, 2004). Cb-HB-NLC kinetics correspond to a hyperbola model, although it's mathematically adjustment value is very similar also to first order. The release of HB follows the same kinetics from gel, as when released from the NLC, but slower (Carvajal-Vidal et al., 2019) because in this case, the drug follows a series of events, which includes its release from the NLC and then spreading between the gel matrix, thus slowing the release rate from the total formulation (Joshi and Patravale, 2008). Both, rapid release and sustained release are beneficial for dermal treatment, the first one allows to exert an initial effect while sustained release allows to provide the drug for a prolonged period of time.

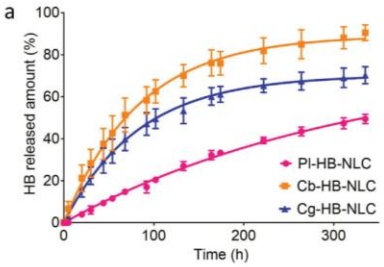
3.6.2. Ex vivo permeation

The permeation study was performed on human skin for 36 h, during this period, the stratum corneum integrity is guaranteed, and so, its ability to act as a membrane is maintained intact (Mallandrich et al., 2017). Fig. 6b shows at 36 h a penetration of 0.06, 0.61 and 0.97 μg for Pl-HB-NLC, Cg-Hb-NLC and Cb-HB-NLC, respectively. Comparatively it is observed that at 24 h. Cb-HB-NLC permeates 1.6 times more than Cg-HB-NLC and 16 times more than Pl-HB-NLC, with an equivalent initial sample concentration, this means that these values are in agreement with the drug's release profiles, in the face of a faster release, faster skin penetration occurs in every measured time, what could indicate that the permeation is mainly determined by HB diffusion from the gels. The results indicate that the drug permeation from the gels is much slower compared to the free drug and even the drug in an HB-NLC suspension (Carvajal-Vidal et al., 2019), which might suggest that the drug stays longer on the skin surface before going into systemic circulation.

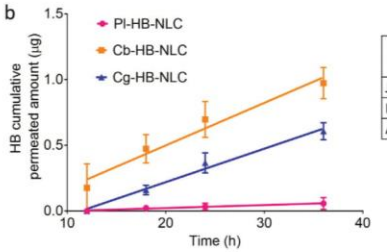
3.7. In vivo and ex vivo tolerance

The results of the Draize's test show that there was no evidence of damage, no edema or erythema produced by direct contact with the formulations, classifying them as non-irritants.

In the histological study (Fig. 7) can be observed that the skin



	Cg-HB-NLC		Cb-HB-NLC		Pl-HB-NLC	
Fit Models	AIC	r^2	AIC	r^2	AIC	r^2
Zero Order	109.155	0.7715	114.471	0.8019	53.533	0.9724
First Order	**72.633**	**0.9733**	90.941	0.9503	**19.373**	**0.9963**
Hyperbola	73.556	0.9718	**90.141**	**0.9526**	21.358	0.9958
Higuchi	89.174	0.9290	96.105	0.9330	55.454	0.9690
Korsmeyer-Peppas	n= 0.439		n= 0.462		n= 0.778	
	87.217	0.937	95.360	0.936	35.641	0.991



	Cg-HB-NLC (max-min)	Cb-HB-NLC (max - min)	Pl-HB-NLC (max-min)
J (µg h^{-1} cm^{-2})·10^3	26.2 (24.4 - 28.9)	32.7 (23.9 - 39.1)	2.4 (1.7 - 2.8)
Kp (cm h^{-1})·10	0.2096	0.2616	0.0192
A36 (µg)	0.61 (0.56-0.68)	0.97 (0.89 -1.11)	0.06 (0.01 - 0.10)

Fig.6. Biopharmaceutical behavior. (a) *In vitro* release profiles of HB from different gels (adjusted to kinetics), (b) *Ex vivo* human skin permeation profile of HB from different gels and permeation parameters. Results represented as mean ± SD.

structures remain organized after application of the gels, there is no significant damage at the histological level between the areas in contact with the formulations in comparison with the control (Fig. 7a). This confirms that the formulations do not incite any disruption in the skin layers after 24 h direct contact, verifying that the mixtures of components used to make the formulations have no negative effect and they are safe for their intended use.

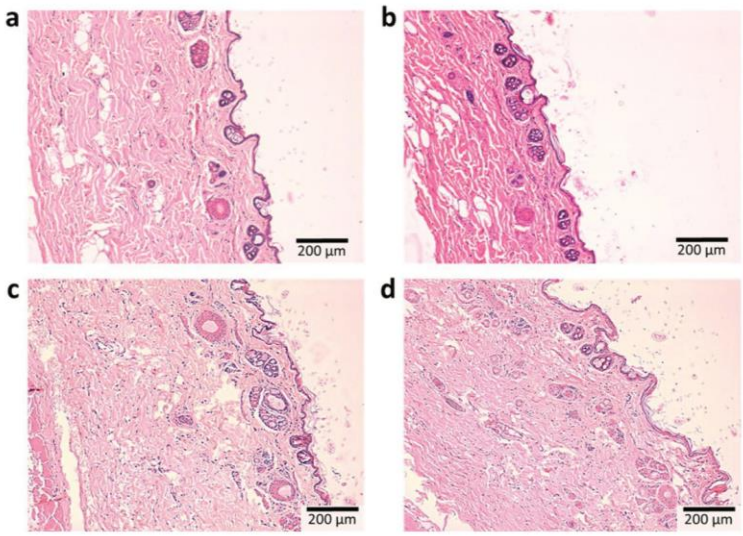


Fig. 7. Histological analysis of rabbits back, blank and in contact areas. (a) No contact skin. Skin in contact with formulation for 24 h (b) Pl-HB-NLC, (c) Cb-HB-NLC, (d) Cg-HB-NLC.

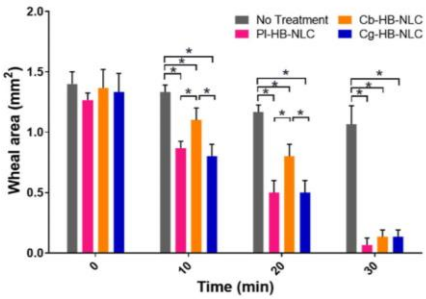


Fig. 8. Efficacy results. *In vivo* histamine-induced wheal suppression test results represented as mean ± SD. Statistically significant differences are represented with * ($p < 0.005$).

3.8. Anti-inflammatory efficacy

In Fig. 8 it can be seen that from the 10 min of the application of the gels, there is an anti-inflammatory effect in comparison with the control. The bleb formation is smaller in every case with a reduction of 35.0%, 17.5% and 40.0% for Pl-HB-NLC, Cb-HB-NLC and Cg-HB-NLC, respectively. At 30 min, it can be seen that the effect does not present statistically significant differences between gels, but the blebs are 87.5% reduced in comparison with the control. These results demonstrate the anti-inflammatory efficacy of the formulations. Pl-HB-NLC shows a constant and evident decrease of the inflammation, probably due to the polymeric matrix, that allows the movement of the HB-NLC out from the formulation, in comparison with Cb-HB-NLC, which, being more viscous and with a thicker matrix, limits the output of HB-NLC, therefore, causing a slower release and a slower anti-inflammatory immediate effect. Considering the permeated parameters obtained in *ex vivo* results, we could inferred that presenting lower values in permeation parameters, would increase the drug time within the skin. A slower drug penetration through the skin would probably be in accordance with a higher drug retention in the skin, which would lead to reaching the drug needed amount to activate the local corticosteroid receptors earlier, which would trigger faster initial local effect.

3.9. Skin integrity parameters (Tolerance in human)

TEWL values indicate no statistically significant differences between the control and Pl-HB-NLC (tendency to decrease of 7.2%), but, the formulations Cb-HB-NLC and Cg-HB-NLC showed a decrease in values ($p < 0.05$) of 9.2% and 21.8% respectively, after 2 h of use (Fig. 9a, b, c). The formulations are well tolerated and it can be considered that they have protective action. Ever since TEWL measures the trans-epidermal water lost, this parameter can be considered as a measure of the integrity of the skin, where a maintenance or a decrease in value is indicative that the tested formulations are suitable for use on skin, since it maintains its barrier properties. On the contrary, an increase in TEWL values would indicate that the evaporation of water through the skin has increased and therefore, the stratum corneum is altered, decreasing its protective barrier function (Akdeniz et al., 2018).

The hydration of the stratum corneum was determined measuring the changes in the dielectric capacity of the skin by corneometry. Fig. 9(d, e, f) shows a statistically significant ($p < 0.05$) increase of 55.7% hydration 2 h after application of Cg-HB-NLC, while Cb-HB-NLC did not show a significant variations with respect to the initial value, but had an increasing tendency of 2.7%. This could be attributed to a possible immediate moisturizing effect of the gels (Bogdan et al., 2017). The hydration decrease presented by Pl-HB-NLC (39.2%) could be attributed to a superficial film formed by the gel, a film with an opaque, dry and rough appearance, visible and evident to the touch that probably acted as an artifact, preventing the correct interaction between the measurement probe and the skin, providing highly diminished hydration values. After application of the formulations, the biomechanical properties indicate that they would have a protective and moisturizing effect by maintaining/decreasing TEWL values and increasing

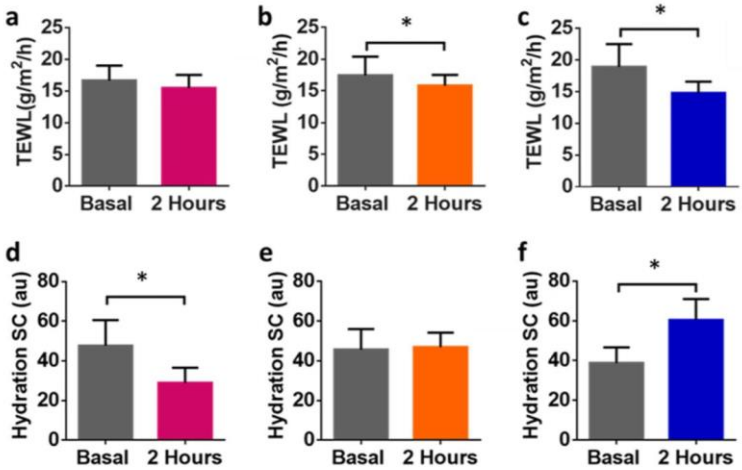


Fig. 9. Skin biomechanical properties. Measures made on 20 healthy volunteers. TEWL determinations: Pl-HB-NLC (a), Cb-HB-NLC (b) and Cg-HB-NLC (c). SC hydration determinations: Pl-HB-NLC (d), Cb-HB-NLC (e) and Cg-HB-NLC (f). Due to the great variability of the skin for these type of measurement, the data does not have a normal distribution. Non-parametric Wilcoxon matched-pairs signed rank was selected as the best statistical test for biomechanical properties analysis. Results represented as mean ± SD.

hydration, respectively, especially for Cg-HB-NLC. A wide variety of skin diseases present dryness and irritation and as a result, it produces itching and scaling of the stratum corneum, getting worse basal disease. The results show that the formulations developed would be a suitable vehicle for HB-NLC, but they could also reduce the physical discomfort associated with the disease, helping to better control the symptoms.

4. Conclusion

The concentration and characteristics of the polymer used in a gel dosage form loaded nanoparticles for topical administration constitutes a key factor to determine if the drug will have immediate or sustained effect, allowing to modulate its release, while acting on the properties of the skin. The results of this research indicate that the Pl-HB-NLC, Cb-HB-NLC and Cg-HB-NLC gels have anti-inflammatory pharmacological effect and are suitable for their use on skin for prolonged periods of time, making possible the administration of the drug in different vehicles that offers the opportunity to select the most appropriate one, according to the skin conditions of a specific patient, not only to treat the underlying disease, but also to reduce the discomfort of the associated symptomatology.

CRediT authorship contribution statement

Paulina Carvajal-Vidal: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Roberto González-Pizarro:** Validation, Formal analysis, Investigation. **Carolina Araya:** Investigation. **Marta Espina:** Formal analysis, Writing - review & editing. **Lyda Halbaut:** Methodology, Validation. **Immaculada Gómez de Aranda:** Investigation. **M. Luisa García:** Conceptualization, Supervision, Writing - review & editing, Project administration. **Ana Calpena:** Conceptualization, Methodology, Formal analysis, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4.

DISCUSIÓN

DISCUSIÓN

La presente investigación tiene por objeto el desarrollo y evaluación de sistemas lipídicos nanoestructurados (NLC) capaces de aumentar la biodisponibilidad de un fármaco esteroideo -HB- a través de las capas de la piel, a fin de ejercer un efecto de prevención o tratamiento antiinflamatorio local con una dosis inferior a la actualmente comercializada.

Por este motivo, se desarrolló un sistema optimizado de NLC conteniendo HB, que posteriormente fue incluido en tres formulaciones semisólidas: gel de carbopol (Cb-HB-NLC), gel de pluronic (Pl-HB-NLC) y cremigel (Cg-HB-NLC). Las formulaciones diseñadas se evaluaron tanto respecto a sus características fisicoquímicas y propiedades biofarmacéuticas, como en su seguridad, tolerancia y eficacia antiinflamatoria *in vivo* e *in vitro*.

El primer paso para la investigación de las propiedades del fármaco fue el desarrollo y validación de un método instrumental que permitiese la correcta determinación y cuantificación del fármaco presente en la formulación y posterior a su interacción con los diferentes estratos de la piel.

Se prepararon ocho soluciones, transparentes, con pH eudérmico (4.0 - 6.6) conteniendo HB y un 5% de promotor de permeación elegido según sus características y posible afinidad con el fármaco: mentona, nonano, azona, limoneno, ácido linoleico, decanol, cetiol y careno (HB+promotor) en una base de transcutol cuya concentración de HB se determinó por HPLC. El método demostró ser adecuado para la cuantificación del HB contenido en las formulaciones, permeado y retenido en el EC. La formulación HB con 5% de mentona (HB-mentona) presentó los mejores resultados de permeación y eficacia, con características favorables para su aplicación

directa en la piel mediante un dispositivo roll-on o spray que facilita la aplicación del producto en zonas específicas para pacientes de cualquier edad con afecciones dérmicas, sin necesidad de tocar el área afectada directamente con las manos (Lulla et al., 2005; Aksoy et al., 2016).

Como bien ha sido descrito, la acción de los corticoides puede ser de alta potencia, por lo que a pesar de ser aplicados por vía tópica, debido a sus características, fácilmente pueden penetrar en la piel y generar efectos adversos a nivel sistémico (Ference y Last, 2009).

Con la finalidad de mejorar las características del fármaco, aumentar su efectividad tópica y reducir su paso a la circulación sistémica, se decidió incorporarlo en un sistema lipídico nanoestructurado de segunda generación, NLC. La fabricación de las HB-NLC se realizó mediante el método de homogenización de alta presión a elevada temperatura, seleccionado por su idoneidad para generar partículas lipídicas de pequeño tamaño y bajo PI con estabilidad adecuada (Akbas et al., 2018). La optimización de las NLC se realizó mediante un diseño de experimentos factorial central compuesto (DoE 2^3). Se utilizaron como variables independientes una mezcla de glicéridos caprílicos (LAS: PEG-8, aceite) y glicerol palmilestarato (Precirol® ATO 5, lípido sólido) en proporción 20:80; un tensoactivo no iónico (Polisorbato: Tween 80®) y cantidades variables de fármaco. Los componentes que conforman las NLC fueron elegidos en base a su seguridad para uso humano y su capacidad de solubilizar el fármaco, requisito esencial para generar una matriz homogénea que ayude a la estabilización del fármaco en la suspensión acuosa (Schäfer-Korting et al., 2007; Doktorovová et al., 2010).

DISCUSIÓN

La utilización de un DoE, permite obtener una gran cantidad de información con un mínimo de experimentos, haciendo mucho más eficiente el proceso de identificación y cuantificación de efectos de múltiples variables simultáneamente (Kumar et al., 2012; Weissman y Anderson, 2015). Las variables independientes seleccionadas fueron la concentración total de lípido (cLipid), la concentración de HB (cHB) y la concentración de tensoactivo (cTw), analizando su efecto en el tamaño promedio de partícula (Zav), el índice de polidispersión (PI), la eficiencia de encapsulación (EE) y el potencial Zeta (ZP). Los resultados obtenidos demostraron que las tres variables independientes tienen efecto significativo en las características de las HB-NLC producidas. El diámetro está directamente influenciado por cLipid; el valor absoluto de ZP aumenta con el incremento de cTw mejorando la estabilidad de las partículas mientras que la EE está directamente influenciada por cHB. Los resultados del DoE permitieron seleccionar una formulación optimizada (Zav: 147 nm; PI: 0.18; ZP:-20.7 mV y EE: 94.5%) que contiene 0.125 mg/ml de HB, contenido total de 3% de lípido y 19 mg/ml de Tween 80®. HB-NLC es una población de distribución de tamaño unimodal inferior a 260 nm, con una concentración de 0.01% HB contenido en la matriz, características que indican su idoneidad para la aplicación vía dérmica, permitiendo la llegada del fármaco a la dermis debido a que penetran efectivamente en el EC o en los folículos pilosos (Verma et al., 2003; Mardhiah et al., 2016). Mediante microscopía electrónica de transmisión (TEM), se determinó posteriormente que las HB-NLC tienen forma esférica lisa sin evidencias de agregación y de características similares a las determinadas mediante DLS.

DISCUSIÓN

Los estudios de interacción entre el fármaco y la matriz lipídica (HB - Precirol - LAS), realizadas mediante calorimetría diferencial de barrido (DSC), espectroscopia de infrarrojo con transformada de Fourier (FTIR) y difracción de rayos (XRD) demostraron que el fármaco y la mezcla de lípidos son compatibles. El fármaco se encuentra disuelto en la matriz y no existen evidencias de la generación de ningún nuevo enlace covalente entre ellos, lo que indicaría que el fármaco es capaz de liberarse desde la matriz de NLC manteniendo su actividad farmacológica (Zhuang et al., 2010; Sánchez-López et al., 2016).

Los estudios de estabilidad a corto plazo de HB-NLC se realizaron mediante dispersión múltiple de la luz. Al tratarse de muestras opacas, los resultados se obtienen a partir de los perfiles de retrodispersión de la luz (BS), lo que permite determinar la existencia de factores de desestabilización tales como coalescencia, floculación, precipitación, sedimentación o cremado de la muestra cuando aún, a ojo desnudo son imposibles de detectar (Mengual et al., 1999; Kaombe et al., 2013). El análisis de los perfiles de BS demostró que la suspensión de HB-NLC es estable a 25 °C y 4 °C por un período de uno y dos meses respectivamente. Este resultado se deduce a partir de la diferencia entre el perfil de BS inicial y final. Perfiles de BS con desviaciones superiores al 10% respecto al perfil inicial indican desestabilización de la muestra (Limón et al., 2015). Los perfiles de inestabilidad obtenidos a partir de los dos meses, podrían deberse al fenómeno de sedimentación reversible de las partículas (Mengual et al., 1999a). La determinación paralela de la morfometría (Zav y PI) de HB-NLC puso de manifiesto, que únicamente a 4 °C estos parámetros se mantienen constantes, resultado que podría atribuirse a que a mayores temperaturas,

aumenta la energía cinética del sistema y produciéndose ablandamiento de los lípidos, provocando colisiones y agregación o floculación de las partículas (Gonzalez-Mira et al., 2011). Considerando entonces los resultados de BS y morfometría, la temperatura de almacenamiento óptima para HB-NLC es 4 °C, asegurando que las partículas se encuentran en estado sólido.

Los estudios biofarmacéuticos de liberación *in vitro* demostraron que HB-NLC y el fármaco libre (equivalente a HB en transcutol), presentan una cinética de liberación de orden uno, con una liberación más sostenida del fármaco contenido en NLC en comparación al HB libre.

Los resultados de la liberación de HB desde la matriz de NLC muestran una liberación inicial rápida (efecto de estallido) posiblemente por desorción del fármaco de la superficie de la NLC. Seguidamente, existe una disminución en la velocidad de liberación, debida probablemente a una erosión de la matriz o a la difusión del fármaco. En comparación, HB-mentona al no tener una matriz compleja y tratarse de una solución de HB libre, presenta una liberación del fármaco mucho más rápida.

Los estudios de permeación de HB+promotores (8 formulaciones), demostraron que el transcutol se puede utilizar como solvente del fármaco sin presentar acción promotora de permeación. Los mejores resultados de permeación se encontraron en HB-nonano y HB-mentona, donde se aumenta 12 y 30 veces, respectivamente, la velocidad de penetración en comparación con HB-transcutol (control), sin alterar la cinética de penetración de orden uno. Esto significaría que la penetración en ambos casos es únicamente dependiente de la concentración de HB (Espenson, 1981). Los otros

promotores bajo estudio presentaron diferencias menos destacables en comparación al control, probablemente debido a que las características químicas y estructurales del promotor, el mecanismo acelerador de la permeación y la interacción entre fármaco y promotor no eran las adecuadas para HB. De esta información se desprende por tanto, que con HB-mentona el efecto en piel sería inmediato, dependiendo únicamente de las características del fármaco y de la interacción que el promotor y HB tengan con el EC (Kasting, 2001).

En comparación, los estudios de permeación de HB-NLC, demostraron que presenta un período de latencia 40% superior a HB libre, debido probablemente a que al tratarse de un sistema de liberación controlada, necesita como paso previo a la permeación, la liberación del fármaco desde la matriz que lo tiene incorporado (Barzegar-Jalali et al., 2008). Los resultados indican también que el mecanismo de penetración cutánea vendría determinado principalmente por el coeficiente de partición vehículo-piel (P1), sobre los mecanismos implicados en el coeficiente de difusión (P2). Esto podría deberse a la alta lipofilia del HB, que favorece la interacción con la matriz de NLC retrasando su liberación desde la formulación hacia la piel. Podríamos suponer por tanto, que HB-NLC al presentar una liberación y permeación más lenta de HB, generaría un sistema con efecto prolongado en el tiempo, influyendo en la retención de HB en el EC y retardando su paso a la circulación sistémica, disminuyendo por ende, los efectos adversos sistémicos característicos del fármaco (Fang et al., 2008; Larese et al., 2009).

Los estudios de citotoxicidad *in vitro* para HB-NLC demostraron ausencia de toxicidad para queratinocitos (HaCaT) y para monocitos (THP-1) a concentraciones inferiores a $2{,}5 \cdot 10^{-3}$ mg/ml de HB, equivalente a la dilución 1/50 de la formulación. A concentraciones superiores se evidencia una disminución en la viabilidad celular, debida probablemente a la presencia de Tween® (Müller et al., 1997).

Paralelamente, los resultados del estudio de tolerancia *in vivo* realizados bajo Test de Draize en conejos, clasifican a las formulaciones HB-mentona, HB-nonano y HB-NLC como no irritantes y seguras para su uso en piel, resultado previsible debido a que los componentes utilizados para formularlos se encuentran ya aceptados para su uso en humanos (Savla et al., 2017).

La eficacia antiinflamatoria de las formulaciones de HB fue determinada *in vivo* mediante la técnica de inducción de una ampolla de inflamación con histamina en conejos albinos New Zeeland. Si bien existen diferentes técnicas para la evaluación de la capacidad antiinflamatoria de los corticoides, se eligió esta técnica por ser simple, reproducible, fiable y no invasiva en comparación a los otros métodos, permitiendo también emular otras condiciones de la respuesta inflamatoria como enrojecimiento y aumento de la temperatura (Singh y Singh, 1986; Abidi et al., 2010).

En primera instancia se evaluó la eficacia de HB-mentona y HB-nonano en comparación con HB-transcutol. De ellos, HB-mentona presentó la mayor eficacia antiinflamatoria, probablemente debido a que también es la formulación con mayor permeación de HB a las 24 horas, lo que indica un flujo y una velocidad de penetración mayor. Esto significaría que para un

tratamiento con efecto antiinflamatorio rápido, mentona sería el promotor de permeación de elección más efectivo para HB.

La eficacia antiinflamatoria de HB-NLC se midió en comparación con NLC sin fármaco como control (NLC-blank). A todos los tiempos estudiados, se demostraron diferencias significativas entre el grupo control y el tratamiento. Las ampollas generadas en el grupo tratado con HB-NLC eran aproximadamente 35% más pequeñas que en el grupo control, indicando efecto antiinflamatorio de HB-NLC.

Paralelamente, el efecto antiinflamatorio de HB-NLC fue evaluado *ex vivo,* midiendo la expresión génica de IL-6 e IL-8 en piel extraída de conejo; e *in vitro,* midiendo la producción de IL-8 en cultivo celular HaCaT y THP-1. Los resultados *ex vivo* indicaron que las muestras tratadas con HB-NLC presentan una disminución del 52% en la producción de IL-6 y un 10% en la de IL-8. Este resultado fue estadísticamente mayor en queratinocitos HaCaT que en monocitos THP-1, lo que revelaría un efecto local más que un efecto sistémico de la formulación. Para la determinación *in vitro*, los cultivos celulares de HaCaT y THP-1 se estimularon con lipopolisacárido (LPS) y TNFα respectivamente para inducir producción de IL-8. Los resultados mostraron que en ambos cultivos celulares la producción de IL-8 se veía disminuida hasta en un 75% cuando se trataban con HB-NLC en comparación al control (NLC-blank), lo que indica el efecto antiinflamatorio de la formulación. Los resultados de eficacia antiinflamatoria concuerdan entre sí y revelan que con una concentración de 0.01% HB, las formulaciones desarrolladas con apenas 1/5 de la dosis utilizada comercialmente (0.05%) exhiben eficacia antiinflamatoria local. En el caso

de HB-mentona-transcutol, debido a que se promueve la penetración del fármaco a través del EC y para HB-NLC debido a que el fármaco es capaz de liberarse desde la NLC y ejercer la acción intracelular típica de los corticoides.

Como paso siguiente a la optimización de las HB-NLC y después de confirmar su eficacia antiinflamatoria, se buscó la manera de aumentar la estabilidad de la formulación y brindar otras alternativas de tratamiento que consideraran las variadas alteraciones dérmicas que generalmente acompañan a las enfermedades inflamatorias de la piel.

Para esto, se desarrollaron y caracterizaron tres geles conteniendo HB-NLC optimizadas: un gel de pluronic (Pl-HB-NLC), un gel de carbopol (Cb-HB-NLC) y un cremigel (Cg-HB-NLC), evaluando su eficacia antiinflamatoria y su efecto sobre las propiedades biomecánicas de la piel.

La fabricación de los geles se realizó a temperatura ambiente y bajo el procedimiento estándar de incluir el polímero previamente pesado en una solución acuosa, con la salvedad de que la formulación de HB-NLC fue utilizada como la fracción acuosa del gel a la que se agregó directamente el polímero, para formar un gel que mantuviese las características de las HB-NLC originales. Este factor resulta de gran importancia, puesto que es necesario mantener la estabilidad de las HB-NLC, la cual podría verse alterada al utilizar procedimientos que aumenten la deformación de los lípidos, como las altas temperaturas o la centrifugación (Shah et al., 2015).

La caracterización macroscópica y microscópica evidenció geles opacos debido a la presencia de HB-NLC en comparación a los geles tradicionales transparentes. A temperatura ambiente, presentaron pH

eudérmico y alta viscosidad con resistencia a fluir, excepto Pl-HB-NLC que se mantiene líquido a 25 °C y gelifica a 32 °C. Bajo microscopio, Cg-HB-NLC demostró ser una emulsión monodispersa con tamaño de gotícula entre 4 y 5 μm, mientras que Cb-HB-NLC y Pl-HB-NLC mostraron una matriz homogénea y delgada con partículas inferiores a 500 mn. Paralelamente, los estudios de SEM evidenciaron para Pl-HB-NLC una matriz ligeramente laxa formada por el entrecruzamiento de las fibras de polímero.

Los estudios reológicos revelaron que Cg-HB-NLC y Pl-HB-NLC presentan un comportamiento pseudoplástico, caracterizado por una disminución de la viscosidad cuando se aumenta la velocidad de deformación aplicada (Ayoubi et al., 2019). Por otra parte, Cb-HB-NLC presentó un alto comportamiento inicial viscoelástico, caracterizado por exhibir tanto propiedades viscosas como propiedades elásticas frente a un estímulo de deformación (Cai et al., 2016).

La comparación de viscosidad entre geles pudo realizarse en las secciones ascendentes para un valor de velocidad de cizalla de 5 s^{-1}. Siendo respectivamente de mayor a menor viscosidad Cb-HB-NLC (167,70 ± 5,03 Pa · s)> Cg-HB-NLC (22,35 ± 0,40 Pa · s)> Pl-HBNLC (0,60 ± 0,01 Pa · s). Paralelamente, estos parámetros fueron determinados en las HB-NLC antes de ser incorporadas a los geles, mostrando comportamiento de fluido newtoniano y una viscosidad muy por debajo de las obtenidas con los geles.

La viscosidad y la resistencia al flujo que presentan las formulaciones desarrolladas, vendría determinada por el entrecruzamiento de las redes poliméricas formadas, haciendo suponer que las formulaciones más viscosas, presentan entrecruzamientos más rígidos y menos flexibles.

Teniendo en cuenta estas consideraciones, el mayor entrecruzamiento, corresponde a Cb-HB-NLC, seguido de Cg-HB-NLC y Pl-HB-NLC. La extensibilidad de los geles demuestra valores inversamente proporcionales a los de viscosidad, presentando la mayor y menor extensibilidad Pl-HB-NLC y Cb-HB-NLC respectivamente.

La caracterización del *swelling* demostró que los tres geles siguieron una cinética de hinchamiento de orden uno pero a diferentes velocidades. Pl-HB-NLC presentó una completa solubilización a los 4 minutos, mientras que Cg-HB-NLC y Cb-HB-NLC requirieron 24 y 90 minutos respectivamente.

Por otra parte, el proceso de degradación, también presentó una cinética de orden uno, donde la pérdida de peso total tuvo lugar a los 80 minutos para Pl-HB-NLC, a los 60 minutos para Cg-HB-NLC y a las 6.5 horas para Cb-HB-NLC. Conjuntamente fue evaluada la porosidad de los geles, evidenciando que el gel más poroso corresponde a Pl-HB-NLC, seguido en orden decreciente por Cg-HB-NLC y Cb-HB-NLC.

Al analizar simultáneamente los resultados de la caracterización de los geles y considerar el tipo de polímero en cada formulación, los resultados indican que los procesos de hinchamiento y degradación suceden más rápido frente a entrecruzamientos laxos, que permiten la entrada y salida del solvente con mayor facilidad, relacionado también con una alta porosidad en comparación a entrecruzamientos más concentrados y rígidos que retrasan ambos procesos y presentan bajos valores de porosidad, como en el caso de Cb-HB-NLC (Tang et al., 2005). Esto es coherente con los resultados de reología y viscosidad, donde debido al alto entrecruzamiento de la red, Cb-HB-NLC evidencia un comportamiento viscoelástico y mayor

viscosidad, completamente contrario a Pl-HB-NLC y Cg-HB-NLC. Podría considerarse entonces que el responsable principal de todas las características que presenta el gel, es el grado y rigidez de entrecruzamiento de las redes de polímero que lo conforman. De este modo, un gel con alto entrecruzamiento de fibras generará un gel más viscoso, menos poroso, menos extensible y con procesos de swelling y degradación más lentos.

Las formulaciones demostraron ser estables durante un periodo superior a 6 meses de almacenamiento a 4 y 25 °C, pero con tendencia a una mayor estabilidad a los 4 °C, coincidiendo con la temperatura óptima de almacenamiento de las HB-NLC antes de incorporar el polímero. Por su parte, Cg-HB-NLC presentó una mayor inestabilidad a 25 °C en comparación a Pl-HB-NLC y Cb-HB-NLC, probablemente debido a la temperatura, que alteraría la interacción entre los componentes lipídicos y poliméricos promoviendo la inestabilidad de la muestra (Ji et al., 2015).

Teniendo en cuenta estos resultados podría considerarse que un aumento en la viscosidad de la fórmula, debido a la adición de gelificantes, favorece la estabilidad del sistema HB-NLC original, puesto que disminuye el movimiento libre de las partículas, retrasando o incluso evitando los fenómenos de inestabilidad, como la agregación o la sedimentación. (Tohver et al., 2001; Souto y Müller, 2005).

Los estudios microbiológicos a los 6 meses de almacenamiento demostraron que no existía contaminación microbiológica en ninguno de los geles. Al ser fabricadas sin conservantes, esto indicaría que la cantidad de agua libre o que los componentes de las formulaciones son suficientes para retrasar o evitar la multiplicación microbiológica.

Los estudios de liberación *in vitro* demostraron que HB sigue la misma cinética cuando es liberado de los geles y de las HB-NLC, pero más lento. Presentan una liberación inicial explosiva que luego disminuye, tendiendo a una meseta con cinética de orden uno. Este comportamiento podría deberse a las características lipídicas del fármaco, ya que su solubilidad en la matriz polimérica está en desventaja en comparación con la matriz de NLC, retrasando así la difusión de HB desde la NLC y su distribución a través de la matriz polimérica, ralentizando la velocidad de liberación de la formulación total (Roseman, 1972).

Los estudios de permeabilidad son coherentes con los resultados cinéticos, demostrando que una liberación más rápida genera una penetración más rápida a todos los tiempos medidos. Esto es indicativo de que la permeación está principalmente determinada por la difusión de HB desde el gel y la lentitud del proceso podría sugerir que el fármaco se permanece más tiempo en la superficie de la piel antes de avanzar hacia la circulación sistémica, en comparación con el HB libre y el fármaco incorporado en nanopartículas (HB-NLC).

Se comprobó la inocuidad de los geles mediante pruebas de Draize y análisis histológico, que pusieron de manifiesto una óptima tolerancia dérmica de las tres formulaciones, sin evidencias de alteraciones a nivel macroscópico ni microscópico de la piel.

La evaluación *in vivo* de la eficacia antiinflamatoria mediante la inducción de una ampolla de inflamación con histamina, demostró que el efecto antiinflamatorio de los geles es evidente a partir de los 10 minutos posteriores a su aplicación en todos los tiempos evaluados, alcanzando su

máxima diferencia a los 30 minutos con una disminución del 87,5% del tamaño de la ampolla en comparación al control.

Pl-HB-NLC muestra una disminución constante y evidente de la inflamación, probablemente debido a la matriz polimérica, que permite el movimiento del HB-NLC fuera de la formulación, en comparación con Cb-HB-NLC, que al ser más viscoso y con una matriz más espesa, limita la salida de HB-NLC, por lo tanto provoca una liberación más lenta y un efecto antiinflamatorio menos inmediato. Considerando los parámetros de permeación de los resultados *ex vivo* se puede inferir que una penetración más lenta del fármaco a través de la piel desencadenaría una mayor retención en la piel, alcanzando la cantidad de fármaco necesaria para activar antes los receptores de corticoesteroides locales, generando un efecto local inicial más rápido.

La evaluación de los parámetros biomecánicos, medida en voluntarios después de dos horas del uso de los geles, demostró buena tolerancia y acción protectora de la integridad de la piel, manteniendo su función barrera debido a la disminución/mantenimiento de TEWL. Las mejoras percibidas en el TEWL y en la hidratación de la piel resultan muy superiores para Cg-HB-NLC, ya que, al tratarse de un cremigel, aporta mayor cantidad de componentes lipídicos en comparación con las otras formulaciones.

Una amplia variedad de enfermedades dérmicas presentan sequedad e irritación produciendo prurito, descamación del estrato córneo y empeorando la enfermedad basal. Los resultados sobre TEWL e hidratación muestran que los tres geles serían un vehículo adecuado para HB-NLC, a la

vez que también podrían reducir el malestar físico asociado a la enfermedad, ayudando a controlar mejor los síntomas específicos de cada paciente.

5.

CONCLUSIONES

CONCLUSIONES

En esta tesis doctoral, se desarrolló un sistema lipídico nanoestructurado de liberación controlada conteniendo Halobetasol propionato para el tratamiento de afecciones dérmicas inflamatorias de la piel. De la investigación realizada se desprenden las siguientes conclusiones:

- La determinación de HB encapsulado en las nanopartículas y en los estudios de permeación en piel humana, se llevó a cabo por HPLC, demostrando ser el método de análisis adecuado.
- Las soluciones de HB en transcutol conteniendo como promotores mentona y nonano, demostraron buena tolerancia cutánea y eficacia antiinflamatoria inmediata.
- Las HB-NLC optimizadas mediante DoE, exhibieron características fisicoquímicas que las hacen adecuadas para su aplicación dérmica.
- Los estudios biofarmacéuticos indican que la incorporación de HB en HB-NLC produce una liberación del fármaco de forma sostenida y promueve la retención del fármaco en la piel, aumentando la seguridad de uso en tratamientos prolongados.
- Los estudios de citotoxicidad *in vitro* y de tolerancia *in vivo*, demuestran que la formulación HB-NLC, es segura a nivel celular e histológico.
- Las formulaciones desarrolladas conteniendo HB (HB-mentona, HB-nonano, HB-NLC, Pl-HB-NLC, Cb-HB-NLC y Cg-HB-NLC) demostraron buena tolerancia cutánea en los estudios *in vivo*.
- HB-NLC reduce la producción de interleucinas pro-inflamatorias *in vitro*, mecanismo que respalda la eficacia antiinflamatoria *in vivo*.
- Los estudios de eficacia *in vivo* han demostrado que las formulaciones conteniendo HB tienen actividad antiinflamatoria.

CONCLUSIONES

- La incorporación de agentes gelificantes aumenta en más de 6 veces la estabilidad del sistema HB-NLC en comparación a las nanopartículas en suspensión.
- Los geles Pl-HB-NLC, Cb-HB-NLC y Cg-HB-NLC demostraron una excelente tolerancia cutánea y actividad farmacológica antiinflamatoria *in vivo*, formando una nueva estrategia de tratamiento para enfermedades dérmicas inflamatorias.
- Las mediciones biométricas, indican que los geles Pl-HB-NLC, Cb-HB-NLC y Cg-HB-NLC presentan capacidad hidratante y protectora de la piel, permitiendo considerarlos alternativas de vehículo idóneo dependiendo de las condiciones de la piel del paciente
- Cg-HB-NLC presentó los mejores resultados de hidratación y protección de la piel, posicionándolo como alternativa de elección para pieles con sequedad y descamación que cursen un trastorno inflamatorio.
- La incorporación de HB en nanopartículas lipídicas y la posterior inclusión en geles, que permite espaciar la frecuencia de administración y reducir los efectos adversos del fármaco, podría constituir, tras el desarrollo clínico adecuado, una alternativa terapéutica eficaz para el tratamiento de enfermedades inflamatorias de la piel.

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