UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMÁCIA

Nicole Esposto Biondo

ESTUDOS DE PERMEAÇÃO CUTÂNEA COM A TESTOSTERONA: UMA ATUALIZAÇÃO DAS CONDIÇÕES EXPERIMENTAIS EM DIFERENTES MODELOS DE PELE E INVESTIGAÇÃO DO EFEITO DE TERPENOS COMO PROMOTORES DE ABSORÇÃO

Florianópolis

2022

Nicole Esposto Biondo

ESTUDOS DE PERMEAÇÃO CUTÂNEA COM A TESTOSTERONA: UMA ATUALIZAÇÃO DAS CONDIÇÕES EXPERIMENTAIS EM DIFERENTES MODELOS DE PELE E INVESTIGAÇÃO DO EFEITO DE TERPENOS COMO PROMOTORES DE ABSORÇÃO

Dissertação submetida ao Programa de Pós-Graduação em Farmácia da Universidade Federal de Santa Catarina para a obtenção do título de Mestre em Farmácia
Orientador: Prof. Drº. Thiago Caon
Coorientadora: Drª. Débora Fretes Argenta

Florianópolis – SC

2022

Ficha de identificação da obra elaborada pelo autor através do Programa de Geração Automática da Biblioteca Universitária da UFSC.

Biondo, Nicole Esposto

Estudos de permeação cutânea com a testosterona: uma atualização das condições experimentais em diferentes modelos de pele e investigação do efeito de terpenos como promotores de absorção / Nicole Esposto Biondo; orientador, Thiago Caon, coorientador, Débora Fretes Argenta, 2022.

192 p.

Dissertação de mestrado - Universidade Federal de Santa Catarina, Centro de Ciências da Saúde, Programa de Pós-graduação em Farmácia, Florianópolis, 2022.

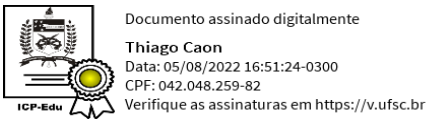
Inclui referências.

1. Farmácia. 2. Permeação. 3. Franz. 4. Terpenos . 5. Testosterona. I. Caon, Thiago . II. Argenta, Débora Fretes. III. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Farmácia. IV. Título.

Nicole Esposto Biondo

Título: Estudos de permeação cutânea com a testosterona: uma atualização das condições experimentais em diferentes modelos de pele e investigação do efeito de terpenos como promotores de absorção

O presente trabalho em nível de mestrado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

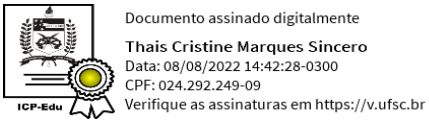


Prof. Thiago Caon, Dr.
Orientador (UFSC)

Profª. Juliana Bidone, Drª.
Membro (UFPel)

Profª. Sara Bianchi, Drª.
Membro (UFRGS)

Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de mestre em farmácia.



Profª. Thaís Cristine Marques Sincero, Drª.
Coordenação do Programa de Pós-Graduação

Florianópolis, 2022.

Este trabalho é dedicado aos meus colegas e
aos familiares queridos.

AGRADECIMENTOS

À minha mãe, Ana Maria, por nunca duvidar de mim, por estar sempre ao meu lado me dando forças quando não sabia mais como seguir.

Ao meu pai, Reinaldo, pela sua constante disponibilidade, alegria e criatividade, sempre com uma ideia genial de como fazer algo novo.

À minha irmã, Rafaela, pela entrega de seu tempo desenhando com carinho todas as figuras com muita dedicação. Obrigada por todo seu apoio sempre.

Ao meu companheiro, Samuel, pela paciência ao longo desses anos, apoio nas horas difíceis, ajuda nos tropeços e risadas para encontrar momentos de paz.

Ao meu orientador, Prof. Dr. Thiago Caon, por toda paciência durante esse trajeto, por me incentivar a não desistir e acreditar na minha capacidade.

À minha coorientadora, Drª Débora Fretes Argenta, pela imensa contribuição para esse trabalho. Muito obrigada pela sua valorosa ajuda, sem a qual nada disso seria possível.

À Bianca, minha colega de mestrado, por todas as horas de conversas e risadas ao longo das horas de limpeza de amostras. Obrigada por me guiar nos prazos e disciplinas sendo meu braço direito nessa etapa.

À Gabriela, por entregar todo seu conhecimento e me ajudar nos experimentos sempre com muita atenção e paciência. Por ter nos recebido com tanto afeto no nosso primeiro dia de mestrado com um gentil presente.

À Clarissa, minha amiga de jornada e da vida, por ser um exemplo de dedicação e persistência nesse caminho acadêmico. Por me inspirar e me orientar tantas vezes nesse processo.

A todos os Professores e colegas do laboratório por terem me auxiliado nos momentos de experimentos e práticas, com paciência e carinho.

A todos os amigos pelas risadas, desabafos, e todo o apoio durante essa jornada.

À UFSC e ao PGFar por viabilizarem este projeto.

A todos aqueles que, de alguma forma, contribuíram para este trabalho e tornaram possível concretizá-lo.

“Existem muitas hipóteses em ciência que estão erradas. Isso é perfeitamente aceitável, elas são a abertura para achar as que estão certas”. (Carl Sagan)

RESUMO

A **primeira etapa** deste estudo envolveu uma análise teórica do impacto de diferentes condições experimentais na taxa de permeação percutânea da testosterona (TST) para ensaios realizados em câmaras de difusão do tipo Franz. Os principais achados foram: (1) a presença de agentes solubilizantes (etanol/propilenoglicol) na fase doadora reduz a quantidade de TST permeada; (2) doses infinitas de TST na fase doadora podem reduzir a taxa de permeação caso eventos de supersaturação levem à precipitação de fármaco; (3) usar preferencialmente pele dermatomizada já que a derme funciona como uma barreira que dificulta a difusão do fármaco para o fluido receptor; (4) priorizar pele suína/humana à pele de rato e peles artificiais (superestimam a quantidade de TST absorvida). Considerando a dificuldade que a TST tem para permear a pele e o uso de promotores químicos de absorção irritantes em formulações do mercado, a **segunda etapa** deste trabalho envolveu ensaios de permeação cutânea *ex vivo* da TST em presença de três terpenos. O tratamento com carvacrol, α-bisabolol e mentol resultou em um raio de reforço da permeação de 7,61, 5,10 e 2,52, respectivamente. O carvacrol melhorou não só a permeação assim como a retenção da TST na pele. Análises espectroscópicas e térmicas revelaram que este agente causa maior desordem nos domínios lipídicos. Além da alta eficácia como promotor de absorção, este agente pode agir de forma sinérgica com a TST no hipogonadismo, no entanto, mais estudos ainda são necessários para comprovar as vantagens desta associação. Por fim, a **terceira etapa** envolveu uma análise comparativa de ensaios de transporte de fármacos realizados em membranas biológicas e não biológicas. Membranas sintéticas e modelos de pele reconstruída possuem como principais limitações: (1) maior taxa de permeação a fármacos que pele humana; (2) ausência de transporte folicular; (3) ausência de metabolismo no caso de membranas sintéticas; (4) diferenças em termos da organização lipídica; (5) são mais afetados por constituintes da formulação tais como promotores químicos. Assim, pele animal e humana ainda deve ser priorizada para ensaios de transporte de fármacos até que novos avanços com modelos de pele reconstruída sejam observados.

Palavras-chaves: permeação cutânea; promotores de permeação; testosterona; célula de Franz.

ABSTRACT

The **first step** of this study involved a theoretical analysis of the impact of different experimental conditions on the percutaneous permeation rate of testosterone (TST) for assays performed in Franz-type diffusion chambers. The main findings were: (1) presence of solubilizing agents (ethanol/propylene glycol) in the donor chamber reduces the amount of TST permeated; (2) infinite doses of TST in the donor chamber can reduce the permeation rate if supersaturation events lead to drug precipitation; (3) dermatomed skin should be preferably used since the dermis works as a barrier that makes it difficult for the drug to diffuse into the recipient fluid; (4) prioritizing porcine/human skin over mouse and artificial skin (they overestimate the amount of TST absorbed). Considering the difficulty that TST has to permeate the skin and the use of irritating absorption chemical enhancers in market formulations, the **second step** of this work involved *ex vivo* skin permeation assays of TST in the presence of three terpenes. Treatment with carvacrol, α-bisabolol and menthol resulted in a permeation enhancement ration of 7.61, 5.10 and 2.52, respectively. Carvacrol improved not only permeation but also the retention of TST in the skin. Spectroscopic and thermal analyzes revealed that this agent causes greater disorder in the lipid domains. In addition to its high efficacy as an absorption chemical enhancer, this agent can act synergistically with TST in hypogonadism; however, more studies are still needed to prove the advantages of this association. Finally, the **third step** involved a comparative analysis of drug transport assays performed on biological and non-biological membranes. The main limitations observed for synthetic membranes and reconstructed skin models were (1) higher drug permeation rate than the human skin; (2) absence of follicular transport; (3) lack of metabolism in the case of synthetic membranes; (4) differences in terms of lipid organization; (5) are more affected by formulation constituents such as chemical absorption enhancers. Thus, animal and human skin should still be prioritized for drug transport assays until further advances with reconstructed skin models may be observed.

Keywords: skin permeation; permeation chemical enhancers; testosterone; Franz-type diffusion cell.

LISTA DE FIGURAS

Capítulo I - Revisão Bibliográfica

Capítulo III

Capítulo IV

LISTA DE QUADROS

LISTA DE TABELAS

Capítulo II

Capítulo III

Capítulo IV

LISTA DE ABREVIATURAS E SIGLAS

ACTH	Adrenocorticotrofina
ARs	*Androgen receptors*
CAEs	*Chemical absorption enhancers*
CF	*Caffeine*
CRH	Hormônio liberador de corticotrofina
DHT	*Dihydrotestosterone*
DSC	Calorimetria exploratória diferencial
ECM	*Extracellular matrix*
EMA	*European Medicines Agency*
EPA	*Environmental Protection Agency*
FDA	*Food and Drug Administration*
FT-IR	Espectroscopia de infravermelho com transformada de Fourier
GnRH	Hormônio liberador de gonadotrofina
GRAS	*Generally recognized as safe*
HPLC	*High-performance liquid chromatography*
ICSH	Hormônio estimulante de célula intersticial
IPCS	*International Programme on Chemical Safety*
Kp	*Permeation coefficient*
LH	Hormônio luteinizante
OECD	*Organisation for Economic Co-operation and Development*
PAMPA	*Parallel Artificial Membrane Permeability Assay*
PBS	*Phosphate-buffered saline*
PG	*Propylene glycol*
SC	*Stratum corneum*
SPE	*Solid-phase extraction*
TEER	*Transepidermal electrical resistance*
TEWL	*Transepidermal water loss*
TST	Testosterona
TWF	*Transepidermal water flux*
WHO	*World Health Organization*

SUMÁRIO

1. INTRODUÇÃO

Estudos *in vivo* em humanos representam o padrão ouro para a análise da permeação cutânea de fármacos (LAI; MAIBACH, 2009), porém, são difíceis de serem realizados e interpretados (ABD *et al.*, 2016). Por este motivo, modelos *in vitro/ex vivo* ainda têm sido priorizados, pelo menos em uma etapa inicial de triagem de diferentes moléculas ou desenvolvimento de novas formulações. Idealmente, peles de tecido humano devem ser selecionadas para estes experimentos, as quais são frequentemente obtidas de cirurgias plásticas ou cadáveres (ESPOSTO BIONDO *et al.*, 2021).

Algumas técnicas utilizadas para a análise da penetração/permeação de fármacos incluem o *tape stripping*, a microdiálise e os testes de permeação *ex vivo* em células de difusão (RANEY *et al.*, 2015). O modelo bicompartimental das células de difusão de Franz ainda é o mais utilizado já que é capaz de fornecer vários tipos de respostas (taxa de permeação/retenção cutânea) e permite extrair informações a respeito do tipo de transporte do fármaco através da pele. Estas células podem ser encontradas nas configurações vertical ou horizontal, estática ou dinâmica (fluxo). Célula vertical e estática são as mais comuns (AULTON; TAYLOR, 2016).

Diferenças na taxa de absorção/permeação podem ser observadas dependendo da região do corpo selecionada (ABD et al., 2016; ROUGIER; LOTTE; MAIBACH, 1987), composição do tecido (quantidade de camadas de células, composição lipídica e proteica), quantidade de apêndices por unidade de área (pêlos e glândulas), hidratação, espessura (MARRAKCHI; MAIBACH, 2007; SANDBY-MØLLER; POULSEN; WULF, 2003) e presença de cicatrizes (MILLS; MAGNUSSON; CROSS, 2006). Assim, a extrapolação dos resultados de estudos deve ser realizada com cautela (WALTERS; ROBERTS, 2002).

Há um número expressivo de estudos de permeação cutânea com a testosterona (TST) na literatura. A TST é o hormônio é responsável pelo desenvolvimento dos órgãos sexuais masculinos durante a gestação. Na adolescência, é responsável pelo desenvolvimento das características físicas secundárias masculinas e, na vida adulta, realiza a manutenção destas (KIM; LEE; KIM, 2000; LEE; CHANG, 2003; NICOLAZZO et al., 2005). Ainda, é responsável pela formação da matriz óssea (BAIN, 2008) e tem ação anabolizante nos músculos (CIGARRÁN et al., 2013).

A produção deste hormônio varia durante o dia, com uma maior concentração entre 6 e 8 h da manhã. Ao longo do dia, observa-se uma queda progressiva dos níveis hormonais seguindo-se um padrão circadiano (BHASIN et al., 2010). No decorrer da vida e principalmente após os 40 anos de idade, a produção da TST começa a diminuir, com uma taxa anual de declínio de 0,4 a 2% (DOHLE et al., 2012), que pode levar ao hipogonadismo. Esta patologia ocorre quando se observam sintomas clínicos sugestivos da doença (ex.: impotência sexual, diminuição da libido, cansaço, depressão do humor, desmineralização óssea, perda muscular, deficiência imunológica e dificuldade no metabolismo de carboidratos), concentração plasmática de TST abaixo de 300 ng/dL e níveis de TST livre abaixo de 6,5 ng/dL (DOHLE *et al.*, 2012; MARTITS; COSTA, 2005).

Assim, várias formulações a base de TST têm sido desenvolvidas com o propósito de restaurar os níveis fisiológicos, incluindo testes em diferentes rotas de administração (MILLS; MAGNUSSON; CROSS, 2006). Embora a rota oral seja priorizada na maior parte dos casos devido a sua conveniência e alta adesão terapêutica que pode ser alcançada, a TST sofre alta metabolização hepática após administração oral (KAUFMAN, 2004). Modificações estruturais tem sido propostas para retardar a sua degradação hepática, porém, derivados sintéticos propostos até o momento tem mostrado uma série de efeitos colaterais indesejáveis (HOTLINE, 1998; KAUFMAN, 2004). Formulações injetáveis, por sua vez, provocam desconforto e dor na aplicação. Além disto, resultam em um rápido aumento da concentração plasmática a níveis superiores ao fisiológico, o que pode resultar em transtornos de humor (ARVER et al., 1997; JOCKENHÖVEL, 2004; LEICHTNAM et al., 2006; NIESCHLAG; BEHRE, 1998). Implantes subcutâneos foram desenvolvidos para evitar esta flutuação da concentração plasmática do hormônio com redução dos efeitos adversos (KAUFMAN, 2004), mas a aplicação requer pessoal treinado. Em vista deste cenário e as vantagens obtidas com o implante subcutâneo, sistemas transdérmicos começaram a ser desenvolvidos. Esta rota de administração não é invasiva, não requer profissional especializado para a aplicação, é indolor, e permite controlar de forma efetiva a liberação do fármaco. Como consequência, há uma maior adesão do paciente ao tratamento (BAERT et al., 2012).

No delineamento de formulações transdérmicas, deve-se analisar as propriedades físico-químicas do fármaco tais como solubilidade, pk_a e log P a fim de definir a

composição da formulação. O fármaco deve ser capaz de ultrapassar regiões lipídicas do estrato córneo, a principal barreira da pele, bem como regiões hidrofílicas da epiderme até o alcance da circulação sanguínea. Candidatos ideais para esta rota de administração devem apresentar valores de log P intermediário (entre 1 e 4), reduzida massa molar (menor que 500 g/mol) e ponto de fusão menor que 250ºC. É desejável também que o fármaco apresente um tempo de meia-vida intermediário que facilita a manutenção das doses do fármaco (AULTON; TAYLOR, 2016).

A TST é um hormônio relativamente lipofílico, com um coeficiente de partição óleo/água (log P) entre 3,32 e 3,42 e massa molar de 288,42 g/mol (HADGRAFT; LANE, 2015; KIM; LEE; KIM, 2000; MISRA et al., 1996). Sua solubilidade em água é de 0,039 mg/mL a 37°C (OKIMOTO; RAJEWSKI; STELLA, 1998). Ainda que a TST apresente baixa massa molar e log P intermediário, observa-se baixa permeação cutânea devido a alta afinidade pela pele e presença de receptores específicos no tecido cutâneo que metabolizam esse hormônio. Por outro lado, baixas concentrações plasmáticas são requeridas para um efeito terapêutico, o que torna a rota transdérmica atrativa. A TST é ainda capaz de chegar até a circulação sistêmica ou se depositar nas células de gordura, formando uma espécie de reservatório do fármaco, gerando uma liberação lenta e gradual na circulação sanguínea (ČEPONIS et al., 2017; WANG et al., 2000).

Considerando a natureza lipofílica e os vários estudos de absorção com essa molécula, esta dissertação apresenta dois estudos de revisão que trazem análises das condições experimentais e o uso de diferentes membranas de pele em ensaios de permeação cutânea (capítulos II e IV). Além disso, tendo em vista as propriedades de barreira do estrato córneo e a necessidade de inclusão de promotores de permeação em formulações transdérmicas (PABLA; ZIA, 2007), a promoção da permeação/retenção da TST com o uso de terpenos foi avaliada (capítulo III). Estes agentes promotores podem agir rompendo a estrutura lipídica intercelular entre os corneócitos, fluidizando o estrato córneo; interagir com as proteínas do meio intercelular, melhorando a partição da molécula; agir nas conexões dos desmossomos entre os corneócitos ou alterar a atividade metabólica do tecido. Todos estes mecanismos podem modificar o arranjo estrutural do tecido e, consequentemente, diminuir as propriedades de barreira do estrato córneo (SILVA *et al.*, 2010; HERMAN; HERMAN, 2015; WILLIAMS; BARRY, 1991), aumentando a difusão da molécula-alvo. O mecanismo preferencial

dependerá das propriedades físico-químicas do fármaco e do promotor (GHAFOURIAN *et al.*, 2004).

Os terpenos foram selecionados como agentes de permeação dada a origem natural que reduz chances de irritação ou toxicidade. Os terpenos são reconhecidamente seguros pela FDA (GRAS, *generally recognized as safe*), fato extremamente relevante já que muitos destes agentes podem penetrar a pele e alcançar a corrente sanguínea (SAPRA; JAIN; TIWARY, 2008; SONGKRO; RADES; BECKET, 2009a). Além de serem rapidamente metabolizados e excretados (HERMAN; HERMAN, 2015), terpenos apresentam atividades farmacológicas específicas (WILLIAMS; BARRY, 2012), o que traz vantagens na sua utilização. O principal mecanismo de ação dos terpenos é a alteração da fluidez do estrato córneo através da interação com os lipídeos intercelulares (HERMAN; HERMAN, 2015; JIANG et al., 2017). Os terpenos selecionados para este estudo foram o alfa-bisabolol, o carvacrol e o mentol, os quais foram solubilizados em propilenoglicol, um cossolvente que age sinergicamente com os terpenos favorecendo a permeação de moléculas (ALAN ANDERSEN, 1999; BARRY; WILLIAMS, 1989; CHEN *et al.*, 2016; SONGKRO; RADES; BECKET, 2009a).

1.1. OBJETIVOS

1.1.1. OBJETIVO GERAL

Analisar como diferentes variáveis do ensaio de permeação afetam a taxa de transporte da TST através da pele humana e animal bem como em membranas não biológicas, além da avaliação do efeito de terpenos como promotores químicos de absorção.

1.1.2. OBJETIVOS ESPECÍFICOS

- ✓ Identificar as principais variáveis do ensaio de permeação cutânea que afetam a taxa de transporte de fármacos com limitações biofarmacêuticas (baixa solubilidade ou permeabilidade) a partir da análise de estudos com a TST;
- ✓ Realizar ensaios de permeação da TST em diferentes tempos utilizando células de difusão de Franz e pele humana;
- ✓ Selecionar, a partir da literatura, terpenos com reconhecido efeito promotor na absorção de compostos lipofílicos;
- ✓ Analisar o impacto dos terpenos nos parâmetros fluxo e coeficiente de permeação da TST durante seu transporte na pele;
- ✓ Monitorar o impacto do tratamento com os terpenos na distribuição da TST na derme e epiderme (estudos de retenção cutânea);
- ✓ Investigar as interações entre os terpenos e estruturas da pele por espectroscopia de infravermelho com transformada de Fourier (FT-IR) e calorimetria exploratória diferencial (DSC);
- ✓ A partir do fluxo de permeação encontrado, realizar o cálculo teórico da quantidade de TST que alcança a circulação sistêmica.
- ✓ Comparar o desempenho de modelos biológicos e não biológicos de pele em ensaios de transporte de fármacos selecionando a cafeína e TST como modelo de fármaco hidrofílico e lipofílico, respectivamente.

2. CAPÍTULO I - REVISÃO BIBLIOGRÁFICA

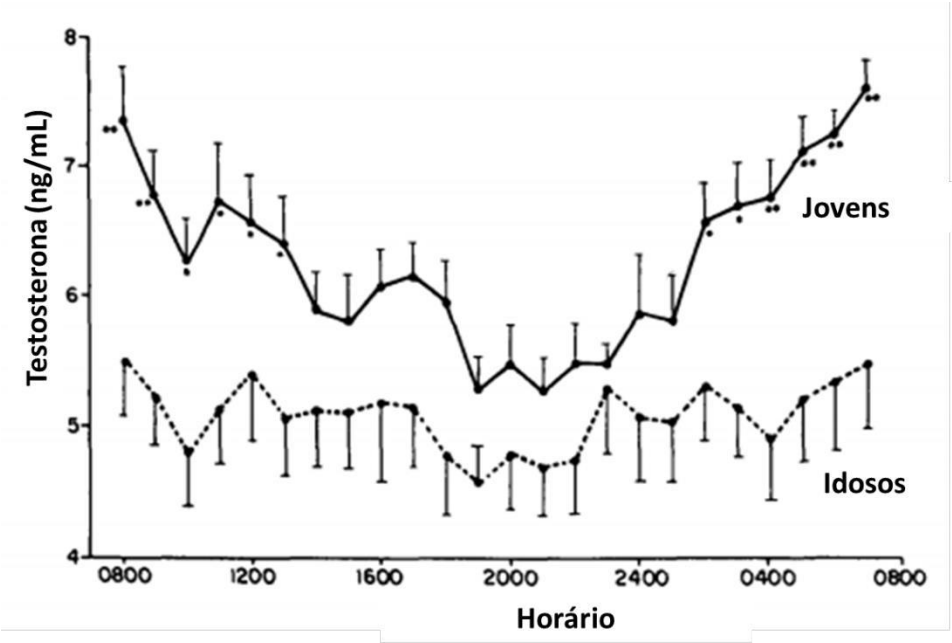
2.1. PROPRIEDADES FISIOLÓGICAS DA TESTOSTERONA

A testosterona (TST) é o principal hormônio andrógeno presente na circulação sanguínea de homens. Atua nas células-alvo através de seu metabólito ativo, a di-hidrotestosterona, gerado pela enzima 5α-redutase (RANG et al., 2016). É produzida a partir do colesterol nas suprarrenais e principalmente pelas células de Leydig dos testículos (NIESCHLAG; BEHRE, 1998). As mais de 500 milhões de células de Leydig são responsáveis pela produção de 6 a 7 mg de TST a cada dia, o equivalente a 95% do total de TST produzido (COFFEY, 1988). Na literatura, há relatos de que a produção não se limita a glândulas, podendo também ser produzida em pequenas quantidades por células cerebrais (BAULIEU, 1997). Embora esta produção seja insignificante perto da quantidade total disponível na circulação sanguínea, acredita-se que seja responsável por algum efeito fisiológico local (NIESCHLAG; BEHRE, 1998).

Nas suprarrenais, o estímulo é derivado dos hormônios CRH (hormônio liberador de corticotrofina) e a ACTH (adrenocorticotrofina) originários do eixo hipotálamo-hipófise-adrenal. Já a produção da TST nas células de Leydig é estimulada pelo ICSH (hormônio estimulante de célula intersticial), também chamado de LH (hormônio luteinizante). A liberação do LH ocorre via adeno-hipófise que, por sua vez, é estimulada pelo GnRH (hormônio liberador de gonadotrofina) produzido no hipotálamo (DOHLE *et al.*, 2012; RANG *et al.*, 2016). Esses hormônios são capazes de regular a quantidade de TST disponível no sangue, bem como a proliferação e diferenciação das células de Leydig (CHEMES, 1996). Na corrente sanguínea, quase toda a TST está ligada a proteínas plasmáticas, especialmente a globulina ligante de esteroides sexuais (DOHLE *et al.*, 2012; RANG *et al.*, 2016).

A produção do hormônio varia durante o dia, iniciando por volta da meia noite com uma maior concentração entre 6 e 8 h da manhã. A concentração cai progressivamente ao longo do dia em um padrão circadiano (BHASIN *et al.*, 2010). A concentração de TST varia durante o dia devido à liberação pulsátil de LH na corrente sanguínea (BRIDGES *et al.*, 1993). Na figura 1, é possível acompanhar a variação da concentração sanguínea da TST ao longo do dia em homens jovens e adultos.

Figura 1. Ritmo circadiano da TST no sangue de homens.



Fonte: traduzido de Bremner & Vitiello & Prinz (1983)

Sua concentração plasmática fisiológica deve estar entre 300 e 800 ng/dL (SNYDER, 2018). Para manter a concentração nos níveis adequados, é necessário haver um equilíbrio entre a produção, o metabolismo e a excreção desse hormônio (NIESCHLAG; BEHRE, 1998).

A TST é responsável pelo desenvolvimento dos órgãos sexuais masculinos durante a gestação. Na vida adulta, mais precisamente na adolescência, é responsável pelo desenvolvimento das características físicas secundárias masculinas, bem como manutenção destas características ao longo da vida (KIM; LEE; KIM, 2000; LEE; CHANG, 2003; NICOLAZZO *et al.*, 2005). Nos ossos, a TST é responsável por duas funções complementares. Após ser convertido a estradiol pela enzima aromatase, inibe a atividade dos osteoclastos, responsáveis pela remodelação óssea, e, portanto, inibe a reabsorção óssea. Também atua via enzima 5α-redutase, que a transforma em di-hidrotestosterona, estimulando osteoblastos - células ósseas responsáveis pela formação da matriz óssea (BAIN, 2008). Ainda nos ossos, possui ação estimulante da hematopoiese, aumentando o hematócrito. A TST atua se ligando em receptores citoplasmáticos de células renais, sendo carreada para o núcleo onde incentiva a ação da RNA polimerase resultando na produção de eritropoietina e sua consequente secreção

(SHAHANI *et al.*, 2009). A eritropoietina é o hormônio regulador da hematopoiese, ou seja, a formação de glóbulos vermelhos. É devido a esta ação que o hematócrito deve ser acompanhado em pacientes que fazem uso da TST (BAIN, 2008).

Nos músculos, a testosterona atua como um hormônio anabolizante que promove hipertrofia (CIGARRÁN *et al.*, 2013). Auxilia na absorção de nitrogênio, que contribui para a formação e manutenção dos tecidos musculares (BAIN, 2008). Os músculos são originados a partir de células multinucleadas presentes em uma matriz de colágeno e nervos motores. Estas células satélites pluripotentes são responsáveis por se diferenciarem em mioblastos e, consequentemente, nas fibras musculares. A TST atua nestas múltiplas etapas do desenvolvimento das fibras musculares (HERBST; BHASIN, 2004). A falta desse hormônio é responsável pela diminuição da massa e força muscular. Além disso, contribui para o aumento da gordura corporal, aumentando o risco de doenças coronarianas e diabetes (BAIN, 2008). Há evidências de que o sedentarismo associado à obesidade esteja relacionado com a baixa concentração de TST em homens, indicando que o hipogonadismo esteja crescente na sociedade (BROWN, 2008).

Existem ainda algumas evidências da ação hormonal da TST no cérebro. Uma delas é a presença de receptores de di-hidrotestosterona e estradiol no cérebro, os quais são metabolitos da 5α- redutase e aromatase, respectivamente. Também há evidências da promoção de crescimento de células neuronais devido a esse hormônio, além de ser responsável pelo aumento da perfusão cerebral em regiões ligadas ao planejamento estratégico, funções motoras, cognição, emoções, concentração e memória (AZAD *et al.*, 2003; BAIN, 2008). Com relação ao humor e à depressão, estudos demonstram uma relação direta da baixa concentração de TST em homens de idade mais avançada com a depressão, embora estudos adicionais ainda se mostram necessários (BAIN, 2008).

Alguns estudos também relatam que a alta incidência de reumatismo em homens de idade mais avançada é decorrente da queda da biodisponibilidade da TST neste grupo. Isto é justificado pelo fato de os hormônios andrógenos serem capazes de abafar as respostas imunológicas hormonais e celulares atuando como uma espécie de anti-inflamatório natural (BAIN, 2008; CUTOLO, 2000).

Acredita-se também que a TST é um dos hormônios responsável pelas características distintas entre homens e mulheres com relação aos comportamentos e

habilidades já que pode agir no Sistema Nervoso Central (HUTCHISON, 1991). No período neonatal, a TST contribui para o desenvolvimento cerebral das células indiferenciadas, atuando na região pré-orbital e no sistema límbico (amigdala) (BETTINI *et al.*, 1992; BRAIN; HAUG, 1992). Uma vez que estas regiões são ativadas, observam-se alterações comportamentais, particularmente durante a puberdade, a qual é caracterizada por um aumento da concentração circulante deste hormônio (BECKER; BREEDLOVE; CREWS, 1992). Embora haja restrições éticas na manipulação dos níveis hormonais humanos para estudos comportamentais e da função cerebral, sabe-se que esta influência é muito maior em animais que em humanos (NIESCHLAG; BEHRE, 1998).

A forma cristalina da TST foi primeiramente isolada de testículos de búfalos em 1935. E, neste mesmo ano, já foi sintetizada quimicamente. A partir daí, começou a ser utilizada clinicamente no tratamento do hipogonadismo sem qualquer comprovação científica (BUTENANDT; HANISCH, 1935; DAVID *et al.*, 1935; NIESCHLAG; BEHRE, 1998). Atualmente, a utilização de formulações a base de TST para o tratamento do hipogonadismo reúne evidências científicas suficientes, com uma diversidade de protocolos terapêuticos.

2.2. HIPOGONADISMO

O hipogonadismo é caracterizado pela deficiência na produção da TST, podendo ser resultante da produção inadequada deste hormônio pelos testículos ou até mesmo uma deficiência na produção dos precursores hormonais no hipotálamo ou pituitária (DOHLE et al., 2012; KIM; LEE; KIM, 2000).

O *hipogonadismo primário* representa a maior parte dos casos e é resultante da baixa produção testicular de TST com consequência na diminuição da espermatogênese e infertilidade. Pode ser resultante da Síndrome de Klinefelter ou tumores testiculares, porém, outras causas também têm sido atribuídas (má formação dos testículos, orquite, doenças virais como caxumba, medicações, toxinas, varicocele, entre outras). O *hipogonadismo secundário*, por sua vez, é derivado de falhas no sistema nervoso central, especificamente no hipotálamo ou na pituitária, sendo uma das principais causas de desenvolvimento tardio na puberdade. O *hipogonadismo adulto*, decorrente da disfunção do eixo hipotalâmico-hipófise, apresenta sintomas das duas categorias

anteriormente mencionadas. Por fim, há o *hipogonadismo por falhas do receptor de andrógeno*, que tem uma frequência mais rara (DOHLE et al., 2012).

Ao longo da vida e principalmente após os 40 anos de idade, a produção da TST começa a diminuir, com uma taxa anual de declínio de 0,4 a 2%. Nos homens de meia idade entre 40 e 79 anos, a incidência do hipogonadismo em análises bioquímicas varia entre 2,1 e 12,8% enquanto que, a incidência de sintomas, varia entre 2,1 a 5,7%. A obesidade, a presença de doenças crônicas e a baixa qualidade de vida representam fatores de risco para o desenvolvimento da doença (DOHLE et al., 2012; MOORADIAN; MORLEY; KORENMAN, 1987).

Os sintomas que podem acometer o homem no hipogonadismo adulto estão descritos no Quadro 1. Dentre estes sintomas, destaca-se a impotência sexual, diminuição da libido, cansaço, depressão do humor, desmineralização óssea, perda muscular, deficiência imunológica e dificuldade no metabolismo de carboidratos (BIZZARRO et al., 1987; DOHLE et al., 2012; LU et al., 2013; MOORADIAN; MORLEY; KORENMAN, 1987).

Quadro 1. Sintomas do hipogonadismo.

Sintomas clínicos sugestivos da deficiência de testosterona
Tamanho diminuído dos testículos
Infertilidade masculina
Diminuição dos pelos do corpo
Ginecomastia
Diminuição da massa corporal e força muscular
Obesidade visceral
Síndrome metabólica
Diabetes tipo II e resistência à insulina
Diminuição da densidade óssea
Anemia branda
Sintomas sexuais
Redução da libido e atividade sexual
Disfunções de ereção
Poucos ou nenhumas ereções noturnas
Sintomas cognitivos e psicológicos
Fogachos
Mudanças de humor, cansaço e irritação
Distúrbios do sono
Depressão
Diminuição das funções cognitivas

Fonte: Dohle *et al.* (2012).

Quando se observam sintomas clínicos sugestivos da doença, concentração plasmática abaixo de 300 ng/dL e níveis de TST livre abaixo de 6,5 ng/dL (DOHLE et al., 2012; MARTITS; COSTA, 2005), é recomendada a reposição hormonal. Para tal, podem-se utilizar formulações nas mais diversas formas de apresentação como a oral, intravenosa, subcutânea, sublingual, nasal, anal ou transdérmica, como será apresentado mais adiante (DOHLE *et al.*, 2012; KIM; LEE; KIM, 2000; RANG *et al.*, 2016).

Muitos pacientes com fraturas vertebrais e osteoporose também apresentam hipogonadismo. Receptores de androgênio são encontrados nos osteoblastos em quantidades significativas para promover a mineralização óssea. Estudos apontam que a reposição hormonal é necessária para a manutenção da densidade óssea (NATIONAL RESOURCE CENTER, 2018; ORWOLL; KLEIN, 1995).

Da mesma forma, o hipogonadismo é também associado a pacientes masculinos infectados com HIV, sendo que 75% deles apresentam os sintomas secundários. As causas aparentam ser multifatoriais e relacionadas a comorbidades. Além disso, alguns medicamentos antirretrovirais apresentam como efeitos adversos a desmineralização óssea. A reposição hormonal é indicada para esses pacientes com a intenção de recuperar a massa muscular e diminuir a osteoporose (BEDIMO *et al.*, 2012; WONG; LEVY; STEPHENSON, 2017).

Segundo um estudo de meta-análise de 2005, os efeitos adversos mais relevantes do uso terapêutico da TST incluem o aumento do hematócrito e eventos prostáticos. Associação com câncer de próstata, problemas cardiovasculares e apneia do sono são mencionados, contudo, ressalta-se a necessidade de comprovação científica (CALOF et al., 2005). Em 2007, uma meta-análise não comprovou o aumento da pressão sanguínea, hiperglicemia e dislipidemia devido ao uso de TST, no entanto, menciona maior ocorrência de riscos cardiovasculares (HADDAD et al., 2007). Na meta-análise de 2010, não foram encontradas diferenças significativas nos eventos de morte, infarto do miocárdio, arritmias cardíacas, diabetes, colesterol LDL, triglicerídeos, pressão arterial e eventos prostáticos (câncer e aumento do índice de PSA) entre usuários e não usuários de TST. Por outro lado, esta análise mais recente relatou queda acentuada nos níveis de colesterol HDL, o que poderia estar associado com doenças cardíacas. Além disto, é relatado o aumento significativo da quantidade de hemoglobina e, consequentemente,

do hematócrito, com aumento do risco de eritrocitose (BHASIN et al., 2003; COVIELLO et al., 2008; FERNÁNDEZ-BALSELLS et al., 2010), que se manifesta com sensação de fraqueza, falta de ar, dores de cabeça, sonolência, zumbido, entre outros (AMARU et al., 2019). Uma meta-análise foi realizada por BORST *et al.* (2014) para verificar a influência da rota de administração nos efeitos adversos. A administração oral mostrou maiores chances de causar problemas cardiovasculares comparativamente a administração transdérmica ou injetáveis.

De acordo com o Guia Prático da Sociedade de Endocrinologia Clínica, homens sob tratamento hormonal devem ser monitorados quanto ao bem-estar, libido, atividade sexual, concentrações séricas de TST, hemoglobina e hematócrito, PSA e exame de toque retal. No primeiro ano, sugere-se um monitoramento após 3, 6 e 12 meses do início da terapia. Nos anos subsequentes, é suficiente uma avaliação anual (DOHLE *et al.*, 2018). É aconselhável o acompanhamento a cada dois anos da densidade mineral óssea (NIESCHLAG, 2015). Como os efeitos cardíacos são questionáveis, a agência norte-americana *Food and Drug Administration* (FDA) menciona que as indústrias farmacêuticas não são obrigadas a incluir nas embalagens/bulas de produtos à base de TST informações relativas a este risco (FDA, 2014).

2.3. FORMULAÇÕES PARA TRATAMENTO

Desde o isolamento e síntese da TST, várias formulações têm sido desenvolvidas com o propósito de repor os níveis fisiológicos da TST em pacientes com hipogonadismo. Uma das principais limitações do tratamento, principalmente nos pacientes que necessitam de administração prolongada de TST, é a via de administração (MILLS; MAGNUSSON; CROSS, 2006).

As formulações orais de TST sofrem um efeito de primeira passagem expressivo, com um curto tempo de meia vida de aproximadamente 10 min. A alquilação da TST permite atrasar a degradação, porém, o derivado sintético é menos potente (KAUFMAN, 2004). Formulações orais de metiltestosterona e oxandrolona também aparecem como alternativas a TST para contornar a baixa biodisponibilidade, mas alta hepatotoxicidade é observada com a administração destes derivados (HOTLINE, 1998). Apesar destas modificações estruturais, muitas destes derivados

ainda são altamente metabolizados, alcançando concentrações sistêmicas ainda insuficientes (KAUFMAN, 2004; MILLS; MAGNUSSON; CROSS, 2006).

A rota sublingual tem sido utilizada para a administração de 17α-metiltestosterona associada a ciclodextrinas, pois sua forma isolada também é caracterizada por alta hepatotoxicidade (NIESCHLAG; BEHRE, 1998). Apesar do retardo na metabolização, o composto ainda foi caracterizado por curta meia-vida (1-6 h), o que requer várias doses diárias. Outra desvantagem é a necessidade de se melhorar a palatabilidade destes sistemas (KAUFMAN, 2004; NIESCHLAG; BEHRE, 1998).

Considerando estas limitações, iniciou-se o desenvolvimento de formulações injetáveis, as quais eram administradas a cada 10 ou 20 dias. Além do desconforto e dor, estas formulações proporcionam um rápido aumento da concentração plasmática a níveis superiores ao fisiológico (ARVER *et al.*, 1997; JOCKENHÖVEL, 2004; LEICHTNAM *et al.*, 2006a). Esta flutuação dos níveis hormonais pode causar transtornos de humor no paciente, além de afetar a libido, atividade sexual e vigor físico (NIESCHLAG; BEHRE, 1998). Devido aos picos de concentração apresentados e efeitos adversos relatados, outros tipos de veículos/rotas de administração continuaram a serem explorados nos anos 80 (KAUFMAN, 2004).

Implantes subcutâneos foram desenvolvidos com o objetivo de manter as concentrações plasmáticas hormonais mais estáveis. Estes dispositivos são introduzidos sob a pele, logo abaixo da epiderme, liberando o hormônio por entre 4 e 5 meses. Ao contrário das injeções intramusculares, esta nova forma farmacêutica evitava flutuações plasmáticas, reduzindo ou até evitando efeitos adversos (KAUFMAN, 2004). Por outro lado, ainda requer profissionais treinados para aplicação da formulação, além do desconforto que se observa após a aplicação.

As vias retal e nasal foram testadas, mas abandonadas devido ao desconforto para o paciente que necessitava de várias aplicações diárias (NIESCHLAG; BEHRE, 1998).

Com o sucesso dos implantes subcutâneos comparativamente a outras vias, várias pesquisas começaram a ser direcionadas para a via transdérmica (NICOLAZZO et al., 2005). Esta rota de administração não é invasiva, nem dolorida, não requer profissional especializado para a aplicação e permite desenvolver sistemas com controle

de liberação. Como consequência, há uma maior adesão do paciente ao tratamento (BAERT et al., 2012). A possibilidade de reduzir a frequência de administração e dose, bem como a necessidade de se aumentar a permeação da TST em curto intervalo temporal representaram os pilares deste novo tipo de formulação (PABLA; ZIA, 2007).

2.4. PREPARAÇÕES TRANSDÉRMICAS

Com o desenvolvimento das formulações transdérmicas, as desvantagens anteriormente citadas foram superadas. Um dos benefícios clínicos descritos é a liberação do fármaco de forma sustentada por longos períodos, já que a administração transdérmica possui cinética de ordem zero, o que mantém a concentração plasmática estável por maior tempo, sem a flutuação apresentada pelas outras formas farmacêuticas (KIM; LEE; KIM, 2000). Além do controle da formulação em si, a própria pele atua como uma barreira que limita a quantidade de fármaco que alcança a circulação sanguínea.

Outra vantagem, como já mencionado, é o fato destas formulações evitarem o metabolismo de primeira passagem. Como consequência, não é mais necessário utilizar altas concentrações de TST devido a sua inativação, o que pode reduzir a ocorrência de efeitos colaterais (BAERT *et al.*, 2012; GHAFOURIAN *et al.*, 2004; LEICHTNAM *et al.*, 2006a). É um tipo de forma farmacêutica que proporciona alta adesão terapêutica devido à facilidade da administração, com a possibilidade de remoção imediata caso se observem efeitos indesejáveis (BAERT et al., 2012; THOMAS; FINNIN, 2004; WILLIAMS; BARRY, 1992). São indolores durante a aplicação e não causam o desconforto de preparações nasais e/ou retais (KANG *et al.*, 2013; KOVÁČIK; KOPEČNÁ; VÁVROVÁ, 2020). Todas estas vantagens justificam o grande número de formulações comerciais atualmente disponíveis para a TST.

As formulações transdérmicas incluem diversos tipos de formas farmacêuticas/veículos. Podem variar entre loções, cremes, unguentos, géis, adesivos, aerossóis e espumas. Em comum, todas estas preparações devem ser capazes de fazer com o que o ativo penetre o estrato córneo, permeando os vários tecidos até alcançar a corrente sanguínea (AULTON; TAYLOR, 2016).

Os cremes, géis e adesivos são os veículos mais utilizados para a TST. Os *cremes* são formulações semissólidas bifásicas constituídas de uma fase oleosa e uma fase aquosa, as quais são estabilizadas por uma agente emulgente. Os *géis*, por sua vez, são sistemas semissólidos em que uma fase líquida está confinada dentro de uma matriz polimérica. Por fim, e não menos importante, há os *adesivos transdérmicos*, que são formas farmacêuticas sólidas que contêm polímeros associados a um sistema aquoso ou um solvente volátil que forma um filme para a aplicação do fármaco (AULTON; TAYLOR, 2016). Os adesivos foram as primeiras formulações transdérmicas desenvolvidas para a TST (KAUFMAN, 2004). Além das vantagens já citadas, adesivos transdérmicos são capazes de liberar uma dose constante e controlada da TST, algo de extrema importância para os casos em que efeitos adversos são sensíveis a variações na concentração plasmática do fármaco (KANG *et al.*, 2013). Por outro lado, podem causar irritação local devido à oclusão do tecido e/ou alta concentração de TST usada. Quando há movimentação vigorosa e/ou excesso de suor (ex.: durante os exercícios), pode ocorrer desprendimento da formulação (JORDAN, 1997; THOMAS; FINNIN, 2004).

Adesivos foram inicialmente desenvolvidos para serem utilizados na bolsa testicular, que possui um estrato córneo mais fino e é um tecido muito vascularizado, diferentemente de outras áreas do corpo. Anteriormente a aplicação, era necessário que os pelos fossem removidos para facilitar a fixação do adesivo, o que gerava desconforto aos pacientes. Além disso, em alguns casos, o tamanho do adesivo era superior ao tamanho da bolsa escrotal, já que muitos pacientes apresentavam hipogonadismo, o que também acabava por reduzir a adesão ao tratamento (CUNNINGHAM, 1989). O Testoderm® foi o primeiro adesivo transdérmico lançado no mercado norte-americano, em 1994, o qual promovia uma liberação diária entre 10 a 15 mg do hormônio (CUNNINGHAM, 1989; KAUFMAN, 2004; NIESCHLAG; BEHRE, 1998). A concentração plasmática máxima é alcançada 2 a 3 h após a aplicação e, quando aplicado pela manhã, pode simular a evolução circadiana natural do organismo (KAUFMAN, 2004). Devido ao local de aplicação e a necessidade de remoção frequente dos pelos, esse tratamento não foi bem aceito pelos pacientes.

Apesar do insucesso dos adesivos escrotais, esta rota de administração continuou a ser explorada pela série de vantagens já citadas. Adesivos transdérmicos aplicados em outras partes do corpo passaram a ser desenvolvidos/estudados. O Testoderm® começou a ser aplicado na região dos braços, costas e nádegas (KAUFMAN, 2004).

Estudos farmacocinéticos foram realizados por Meikle *et.al.* (1996) para entender o impacto da região de aplicação na concentração plasmática da TST. A biodisponibilidade do hormônio foi maior para os adesivos posicionados nas costas, seguido da coxa, braço superior, abdômen, peito e canela. O estudo ressaltou a importância de se utilizar os adesivos nas quatro primeiras regiões a fim de otimizar a absorção do hormônio. Como alguns usuários destes adesivos relataram dermatite e descolamento do adesivo (KAUFMAN, 2004), a indústria continuou a busca por novas formas farmacêuticas que pudessem melhorar a adesão ao tratamento. Frente a isto, géis transdérmicos foram propostos.

Após a administração de gel de TST a 1%, Wang et al. (2000) observaram um rápido alcance da concentração plasmática máxima (30 min após a aplicação), com redução ao longo de 24 h. Após remoção da formulação, os níveis séricos de TST caíram entre 40 e 50% dentro de 48 h. A formulação desenvolvida não causou irritação cutânea e a rápida secagem após a aplicação reduziria a contaminação acidental entre indivíduos.

O Androgel® (Unimed Pharmaceuticals, GA, USA) foi o primeiro gel transdérmico de TST aprovado nos Estados Unidos no ano 2000. Um ano após a sua comercialização, esta forma farmacêutica já passou a ter a preferência dos consumidores em relação aos adesivos (HADGRAFT; LANE, 2015). Disponibilizado comercialmente nas dosagens de 5, 7,5 e 10 g, foi desenvolvido para ser aplicado nos ombros, parte superior dos braços e/ou abdômen, atingindo níveis terapêuticos entre 30 min e 4 h após a aplicação. A estabilização terapêutica ocorre entre 1 e 3 dias da primeira administração (SWERDLOFF et al., 2000). Nesta primeira formulação desenvolvida, apenas 10% da dose total de TST encontra-se biodisponível para a absorção (WANG et al., 2000), abrindo um campo para o aprimoramento de formulações desta categoria.

Três anos depois, lançou-se o gel transdérmico Testim® (Ferring Pharmaceuticals, UK). Em comparação com o Androgel®, possui maior absorção e, desta forma, não podem ser considerados bioequivalentes. Isto se deve a presença do promotor de permeação pentadecalactona (HADGRAFT; LANE, 2015; MCNICHOLAS *et al.*, 2003). Em 2010 e 2014, lançaram-se no mercado os géis Fortesta® e Volexo®, respectivamente (DOBS *et al.*, 2012; HADGRAFT; LANE, 2015).

Como as formas farmacêuticas em gel ficam expostas, diferentemente das formulações adesivas, podem contaminar parceiras e crianças do usuário. Esta contaminação acidental pode levar a efeitos indesejados como amadurecimento da idade óssea com comprometimento do crescimento da criança, aumento dos órgãos sexuais, desenvolvimento de puberdade precoce, aumento de libido e comportamentos agressivos (HADGRAFT; LANE, 2015; PABLA; ZIA, 2007; THOMAS; FINNIN, 2004).

2.5. CARACTERÍSTICAS DESEJÁVEIS PARA FÁRMACOS INCORPORADOS EM SISTEMAS TRANSDÉRMICOS

No delineamento de formulações transdérmicas, deve-se analisar as propriedades físico-químicas do fármaco tais como solubilidade e o pk_a a fim de definir a composição da formulação. Caso este fármaco apresente baixa solubilidade aquosa, por exemplo, agentes solubilizantes podem ser incluídos a fim de evitar fenômenos de separação de fase, precipitação ou problemas de uniformidade de conteúdo. O pk_a, por sua vez, interfere na relação entre espécies ionizadas e não ionizadas, trazendo impactos tanto na solubilidade quanto absorção. A formulação deve se manter estável até o momento da sua utilização, seus componentes devem ser compatíveis entre si e uma quantidade suficiente de fármaco deve ser liberada do sistema. O fármaco deve ultrapassar regiões lipídicas do estrato córneo, a principal barreira da pele, bem como regiões hidrofílicas da epiderme até o alcance da circulação sanguínea. Candidatos ideais para esta rota de administração devem apresentar valores de log P intermediário (entre 1 e 4), baixos valores de massa molar (menor que 500 g/mol) e ponto de fusão menor que 250 ^{o}C. Para adesivos transdérmicos, a dose terapêutica deve ser baixa (menor que 10 mg por dia) ou os fármacos devem ser potentes já que a formulação apresenta dimensões menores (diferente das preparações transdérmicas semissólidas que podem ser aplicadas em uma área maior). É desejável também que o fármaco apresente um tempo de meia-vida intermediário, pois uma metabolização muito alta exigiria altas doses de fármaco na formulação (AULTON; TAYLOR, 2016).

2.6. ASPECTOS BIOFARMACÊUTICOS E CARACTERÍSTICAS FÍSICO-QUÍMICAS DA TESTOSTERONA

A TST apresenta baixa permeação cutânea, o que pode ser associado à sua alta afinidade pela pele e aos receptores específicos existentes no tecido cutâneo. A existência destes receptores se deve à variedade de funções que a TST exerce no organismo, como já mencionado. Os receptores localizam-se nos queratinócitos da epiderme e fibroblastos da derme. A TST é metabolizada a di-hidrotestosterona pela enzima 5α-redutase e a estradiol pela aromatase presente no tecido adiposo e na pele (CHEN; THIBOUTOT; ZOUBOULIS, 2002).

A TST é um hormônio relativamente lipofílico, com um coeficiente de partição óleo/água (log de P) entre 3,32 e 3,42 (HADGRAFT; LANE, 2015; KIM; LEE; KIM, 2000; MISRA *et al.*, 1996). Sua solubilidade em água é de 0,039 mg/mL a 37°C (OKIMOTO; RAJEWSKI; STELLA, 1998). O Quadro 2 e a Figura 2 apresentam informações físico-químicas/farmacocinéticas e a estrutura química da TST, respectivamente.

Quadro 2. Propriedades físico-químicas e farmacocinéticas da testosterona.

Massa molar	288,4 g/mol
Intervalo de fusão	152 a 157°C
Log P	3,32 a 3,42
Solubilidade	0,039 mg/mL a 37°C
Tempo de meia-vida ($t_{1/2}$)	10 a 100 min
Ligação a proteínas plasmáticas	97 a 98%
Clearence renal	1272 ± 168 L/dia

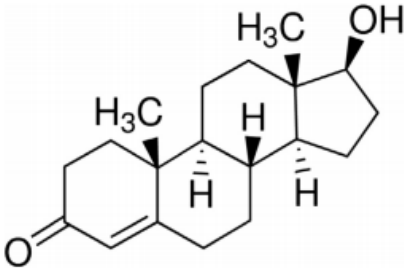


Figura 2. Estrutura química da testosterona.

Fonte: Hadgraft & Lane (2015)

Seu tempo de meia vida varia entre 10 a 100 min no plasma (HELLMAN; ROSENFELD, 1974). Seu grupo 17-OH sofre oxidação hepática, onde é reduzida a di-hidrotestosterona e estradiol. Os metabólitos resultantes ainda sofrem conjugação, glicuronidação e sulfonação para serem secretados pela urina. Apenas 6% do hormônio é excretado na sua forma original (HADGRAFT; LANE, 2015).

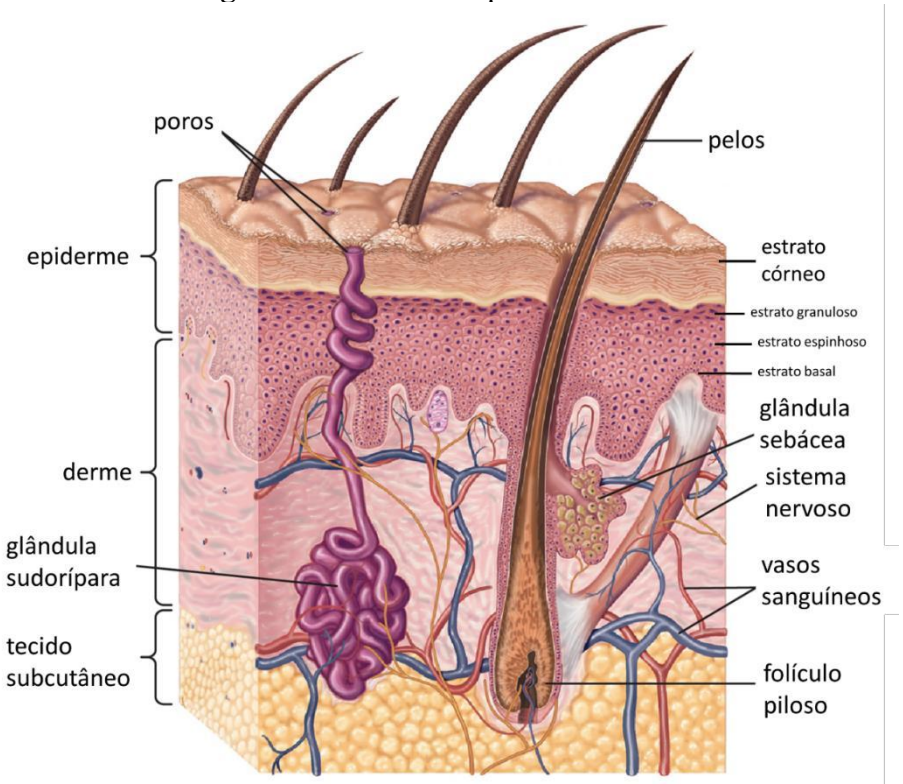
Na circulação sistêmica, a TST encontra-se ligada fortemente a globulinas específicas de ligação de hormônios sexuais e fracamente ligada a albuminas plasmáticas. Apenas 2% é encontrada na forma livre (PARDRIDGE, 1986).

Ainda que a TST apresente baixa massa molar e log P intermediário, observa-se baixa permeação cutânea pelas razões já expostas. Por outro lado, concentrações plasmáticas baixas são requeridas para um efeito terapêutico, o que torna a rota transdérmica atrativa. Ao mesmo tempo em que a alta afinidade do composto dificulta sua passagem pela derme, ao alcançar esta região, a TST é capaz de chegar até a circulação sistêmica ou ainda se depositar nos tecidos subjacentes. Os tecidos subcutâneos apresentam alta quantidade de células de gordura, formando uma espécie de reservatório do fármaco. Com isto, ocorre uma liberação lenta e gradual da TST na circulação sanguínea (ČEPONIS et al., 2017; WANG et al., 2000).

2.7. ASPECTOS ANATÔMICOS E FISIOLÓGICOS DA PELE

A pele é considerada o maior órgão do organismo, com uma área de 1,8 m². É constituída por várias camadas de células (figura 3). Além de exercer propriedades de barreira a xenobióticos e microrganismos (BAERT et al., 2012), contribui para a manutenção da hidratação corpórea e regulação da temperatura corporal (FOX *et al.*, 2011). Sua espessura varia de acordo com o local e a idade do indivíduo (ABD *et al.*, 2016)

Figura 3. Estrutura da pele humana.



Fonte: traduzido e adaptado de Britannica (2018).

A *epiderme* está localizada acima da derme. Na sua composição, aparecem diferentes tipos celulares tais como queratinócitos, melanócitos e células de Langherans (PALMER; DELOUISE, 2016). Os queratinócitos se originam no estrato basal e se diferenciam conforme migram para as camadas mais externas, formando o estrato espinhoso, o estrato granuloso e, por último, o estrato córneo (ABD *et al.*, 2016; AULTON; TAYLOR, 2016). Estima-se que esta diferenciação celular dure aproximadamente 14 dias. No estrato basal, as células apresentam organelas típicas, porém, ao alcançar o estrato córneo, mostram-se anucleadas, achatadas e desvitalizadas (AULTON; TAYLOR, 2016). Por este motivo, a epiderme é subdividida em viável e estrato córneo (ALJUFFALI; LIN; FANG, 2014).

O *estrato córneo* possui de 10 a 15 camadas de células, chamadas de corneócitos, com uma espessura entre 10 e 20 µm (BROWN *et al.*, 2006; ZHANG; FANG; FANG, 2011), que varia dependendo da região do corpo e hidratação da pele. Na sola dos pés e palma das mãos, como são regiões de atrito, há presença de uma camada de células adicional, o estrato lúcido. Ao contrário de outras barreiras lipídicas do corpo, as células do estrato córneo são desvitalizadas e constituídas basicamente por

lipídeos tais como ceramidas (40 a 50%), ácidos graxos (15 a 25%), triglicerídeos (10%), colesterol (25%) e queratina (BAKONYI *et al.*, 2018; CHEN *et al.*, 2016). As ceramidas interconectam os corneócitos resultando em uma estrutura semelhante a uma parede de tijolos - corneócitos preenchidos de queratina seriam os "tijolos" e o "cimento" seriam os lipídeos. As células do estrato córneo permanecem na superfície da pele por aproximadamente 2 semanas até serem substituídas/descamadas (AULTON; TAYLOR, 2016).

A *derme* encontra-se acima do tecido subcutâneo e abaixo da epiderme. Possui uma espessura maior que a epiderme, entre 3 e 5 mm. É constituída de colágeno e elastina banhados por um gel de mucopolissacarídeos formando uma estrutura semelhante a um hidrogel. É nela onde se encontram os anexos da pele (folículos pilosos, glândulas sebáceas e sudoríparas). É a porção metabolicamente ativa da pele por ser altamente irrigada pelos vasos sanguíneos que chegam até 0,2 mm abaixo da superfície, no limite entre a derme e a epiderme. Por esse motivo, é responsável por realizar o controle da temperatura corporal, reparar danos teciduais, bem como realizar a reposição de oxigênio e nutrientes na pele (AULTON; TAYLOR, 2016). Por ser uma camada essencialmente hidrofílica, não interrompe o fluxo da maioria das moléculas, com exceção das excessivamente lipofílicas (KOVÁČIK; KOPEČNÁ; VÁVROVÁ, 2020).

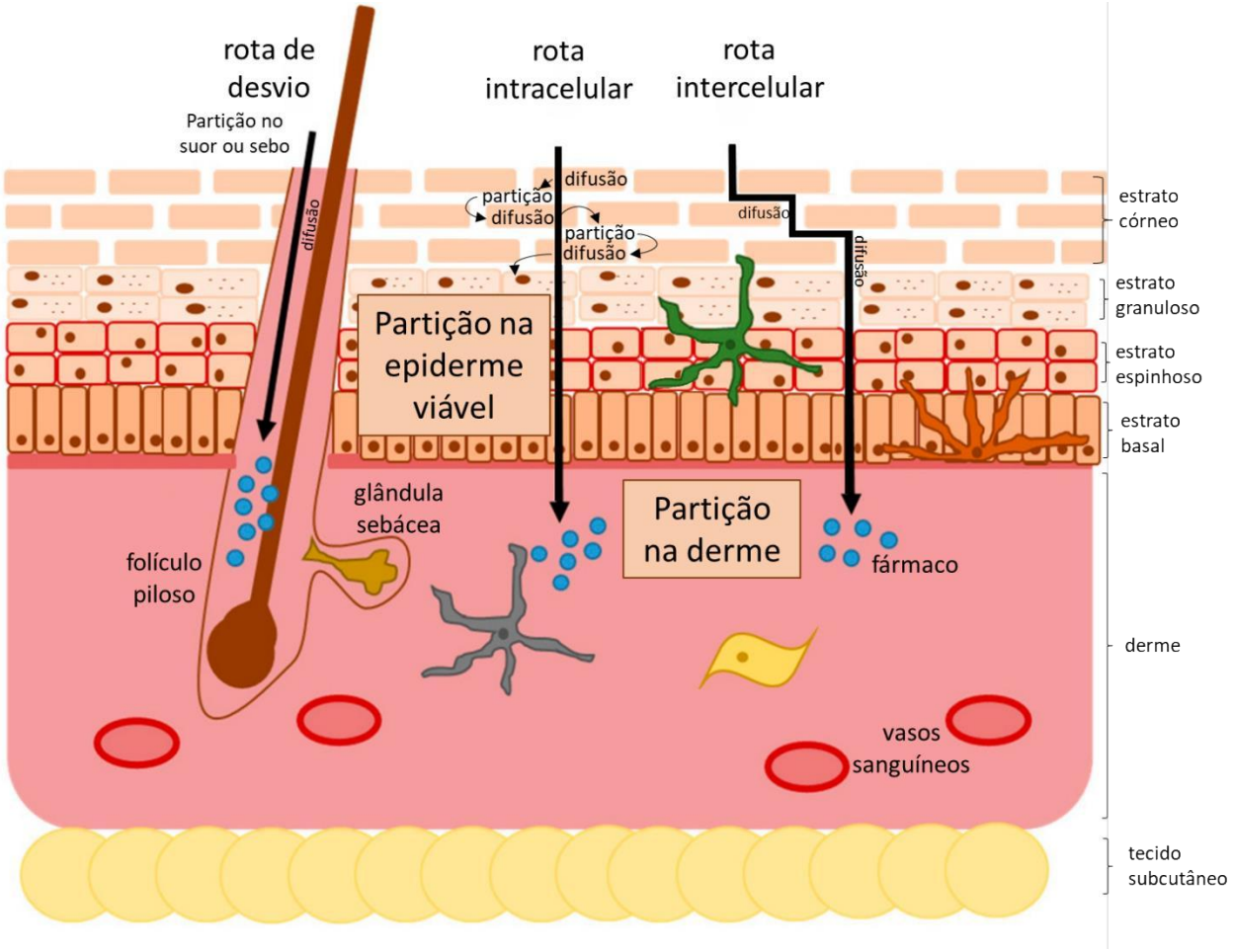
O *tecido subcutâneo* é a camada mais profunda da pele (ABD *et al.*, 2016). É formado por lóbulos de gordura, com uma espessura variável dependendo da localização na superfície corpórea, sendo mais espesso no abdômen e mais fino na bolsa escrotal (IYER *et al.*, 2017). Tem como função a proteção mecânica contra impactos, auxilia na manutenção da temperatura corpórea e estoca energia para as atividades metabólicas (AULTON; TAYLOR, 2016).

Por fim, tem-se os *anexos da pele*, que podem funcionar como "atalhos" para o transporte de moléculas até a corrente sanguínea. Os folículos pilosos estão associados as glândulas sebáceas. Estão presentes entre 50 e 100 folículos pilosos por cm^2 de pele. As glândulas sebáceas são responsáveis pela produção do sebo, o qual é composto por ácidos graxos, ceras e triglicerídeos, que atua lubrificando e auxiliando na manutenção do pH da pele, que é levemente ácido (AULTON; TAYLOR, 2016).

2.8. TRANSPORTE DE MOLÉCULAS ATRAVÉS DA PELE

Na pele, as moléculas podem seguir três caminhos distintos: rotas intracelulares, intercelulares e apêndices – folículo ou glândulas sudoríparas (Figura 4) (ALJUFFALI; LIN; FANG, 2014; HERMAN; HERMAN, 2015). As rotas intra- e intercelulares são ambos processos de difusão passiva, ou seja, o fármaco é transportado a favor de um gradiente de concentração. Na rota intracelular ou transcelular, o fármaco atravessa os corneócitos que compõe o estrato córneo, passando pelo seu interior repleto de queratina. Já na rota intercelular, a difusão ocorre de forma labiríntica, no meio intercelular lipídico entre os corneócitos. As rotas de desvio seguem pelos anexos da pele, difundindo-se pelo suor ou pelo sebo contra o fluxo das glândulas até atingirem o vaso sanguíneo que irriga estas estruturas (KNORR *et al.*, 2009a; PATZELT; LADEMANN, 2013). Compostos hidrofílicos tendem a utilizar as rotas de desvio como os poros e os apêndices ou a rota intercelular. Já os compostos lipofílicos tendem a utilizar as rotas passivas como a intracelular ou transcelular (MITRAGOTRI, 2003; TODO; MOHD, 2017).

Figura 4. Processos que ocorrem durante a liberação transdérmica de fármacos



Fonte: traduzido e adaptado de Palmer & Delouise (2016).

2.9. MODELOS PARA AVALIAÇÃO DA PERMEAÇÃO CUTÂNEA DE FÁRMACOS

Estudos *in vivo* em humanos representam o padrão ouro para a análise da permeação cutânea de fármacos (LAI; MAIBACH, 2009), porém, são difíceis de serem realizados já que exigem autorização de comitês de ética bem como a participação de voluntários. Além disso, as respostas destes ensaios são mais difíceis de serem calculadas e interpretadas por apresentarem grande variação entre os indivíduos (ABD *et al.*, 2016). Por este motivo, modelos *in vitro/ex vivo* ainda têm sido priorizados, pelo menos em uma etapa inicial de triagem de diferentes moléculas ou desenvolvimento de novas formulações.

Idealmente, peles de tecido humano devem ser utilizadas nestes experimentos, as quais são frequentemente obtidas de cirurgias plásticas e de cadáveres, com a devida

aprovação ética de uso. Deve-se ainda levar em consideração que as diferenças de absorção das diferentes partes do corpo (ABD *et al.*, 2016; ROUGIER; LOTTE; MAIBACH, 1987) decorrentes da composição do tecido, hidratação e espessura influenciam nos resultados (MARRAKCHI; MAIBACH, 2007; SANDBY-MØLLER; POULSEN; WULF, 2003).

Algumas técnicas utilizadas para a análise da penetração/permeação de fármacos incluem o *tape stripping*, a microdiálise ou microperfusão *in vivo* e os testes de permeação *ex vivo* utilizando células de difusão (RANEY *et al.*, 2015).

O ensaio do *tape stripping* considera apenas o fármaco presente no estrato córneo, não monitorando a quantidade do mesmo presente nas camadas subjacentes ou corrente sanguínea. Por esta razão, é recomendado para comparar a *performance* de formulações tópicas (ESCOBAR-CHAVEZ *et al.*, 2008; SURBER; SCHWARB; SMITH, 2001). Neste ensaio, fitas adesivas sucessivas são aplicadas no estrato córneo para a remoção dos corneócitos contendo o fármaco. Na sequência, o fármaco é extraído destas células com um solvente específico e dosado por técnicas analíticas que sejam sensíveis o suficiente para detectar a quantidade de fármaco ali presente (SHAH; MAIBACH; JENNER, 2014). O operador deve remover as fitas aplicando sempre a mesma força de modo a garantir maior reprodutibilidade dos resultados. (ABD *et al.*, 2016).

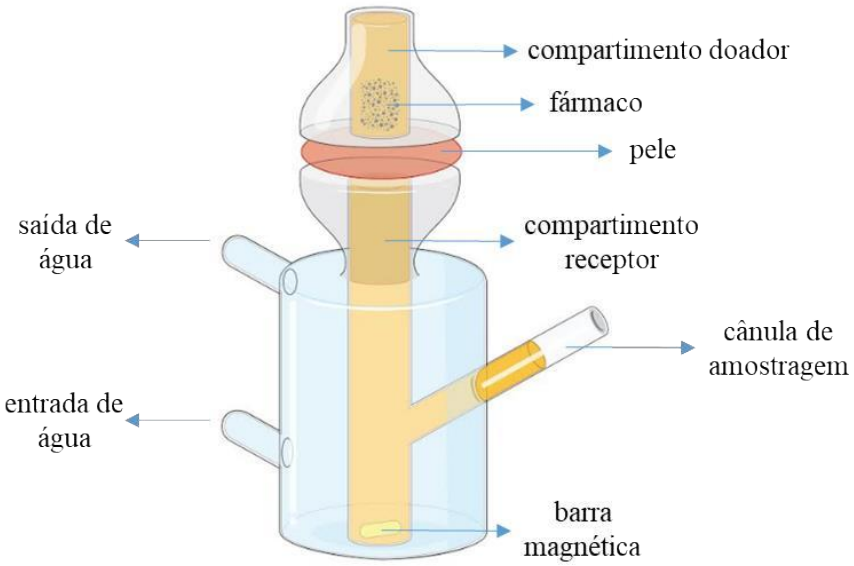
Na microdiálise, uma cânula semipermeável é inserida sob a pele, alcançando a derme. No interior desta cânula, há uma solução de perfusato que mimetiza a circulação sistêmica. Esta disposição deve permitir a troca de substâncias entre o fluido extracelular e a sonda, e assim determinar a concentração local do fármaco para que se possa traçar um perfil de concentração de fármaco ao longo do tempo (ABD *et al.*, 2016). Como desvantagem desta técnica, pode-se destacar a possibilidade do desenvolvimento de inflamações locais que interferem no fluxo dos xenobióticos e moléculas endógenas (STENKEN *et al.*, 2010) e a grande variação pessoal nos resultados interindividuais (BENFELDT *et al.*, 2007).

Como ambos os ensaios mencionados acima apresentam muita variação, o FDA recomenda a utilização dos ensaios *in vitro/ex vivo* devido a maior correlação com os resultados *in vivo*, além da alta reprodutibilidade e precisão. Ensaios em células de difusão utilizando pele humana representam modelos promissores neste sentido já que

conseguem predizer com certa confiabilidade resultados de biodisponibilidade (RANEY *et al.*, 2015). Além disto, este modelo tem sido utilizado para análise de bioequivalência de doses (ABD *et al.*, 2016).

O modelo bicompartimental das células de difusão de Franz (figura 5) é o mais tradicionalmente utilizado nos ensaios de permeação cutânea, o qual pode apresentar diferentes configurações (sistemas horizontal e vertical). No compartimento superior ou doador, é inserida a solução do fármaco ou formulação-teste. Quantidade finitas ou infinitas de fármaco podem ser consideradas. Em geral, doses infinitas são utilizadas para a composição de sistemas transdérmicos. No compartimento inferior, encontra-se o fluido receptor, o qual deve mimetizar o comportamento fisiológico do sangue, manter a integridade do tecido e uma completa solubilização do fármaco. A presença de um agitador magnético neste compartimento visa garantir uma distribuição uniforme do fármaco na solução, impedindo o acúmulo do fármaco na região abaixo da membrana. Além disto, há a presença de uma jaqueta de água externa para garantir um controle da temperatura do experimento, que deve ser de 37 ou 32°C (a depender da literatura consultada). As amostras para análise são retiradas deste último compartimento em intervalos pré-definidos. Por fim, o fármaco é quantificado a fim de se conhecer a quantidade permeada (AULTON; TAYLOR, 2016).

Figura 5. Célula de difusão de Franz.



Fonte: elaborado pelo autor.

Embora a pele humana represente o tecido ideal para este tipo de ensaio, limitações relativas a disponibilidade de material e questões éticas tem contribuído para uso de tecidos de animais como suínos, ratos, camundongos, porquinho da índia e cobras (ABD *et al.*, 2016; HERMAN; HERMAN, 2015). Por outro lado, a composição da pele (quantidade de camadas de células, composição lipídica e proteica), espessura das camadas que a compõe, porosidade e irrigação sanguínea varia entre as espécies. Além disto, observam-se diferenças relativas à quantidade de apêndices por unidade de área (pêlos e glândulas). Alguns animais possuem mais pêlos do que o tecido cutâneo humano (GODIN; TOUITOU, 2007; HUEBER; SCHAEFER; WEPIERRE, 1994; LIN *et al.*, 1992; MONTEIRO-RIVIERE, 1991). Cicatrizes como estrias também afetam a permeação cutânea devido a diferenças na composição da pele (MILLS; MAGNUSSON; CROSS, 2006). A extrapolação dos resultados de estudos nos diversos tipos de pele apresenta limitações e deve ser analisada com cuidado (WALTERS; ROBERTS, 2002). A aplicação de uma mesma formulação em diferentes partes do corpo humano também pode resultar em diferenças na quantidade absorvida (ORIBA; BUCKS; MAIBACH, 1996).

Como alternativa às membranas biológicas, há as membranas sintéticas e os modelos de pele reconstruída. As membranas sintéticas são compostas por acetato de celulose, dimetilpolissiloxano, hidrocarbonetos, álcoois de cadeia longa, miristato de isopropila, lipídios cutâneos (OTTAVIANI; MARTEL; CARRUPT, 2006), colesterol, ácidos graxos livres e análogos de ceramidas (ceramidas sintéticas, diamidas de ácido tartárico de cadeia longa) (SINKÓ *et al.*, 2012). Essas membranas podem ser colocadas na interface das câmaras de difusão ou preparadas diretamente no aparato PAMPA (*Parallel Artificial Membrane Permeability Assay*). O ensaio PAMPA é geralmente utilizado para uma triagem rápida de fármacos transportados passivamente através da pele. Nele, uma membrana lipídica artificial separa os compartimentos doador e receptor em placas de 96 cavidades (NEUPANE *et al.*, 2020). Já nos modelos de pele reconstruída, as camadas de células humanas são geralmente cultivadas em uma matriz polimérica. Diferentes tipos de células podem ser considerados para alcançar uma estrutura com a composição e complexidade desejadas. Modelos reconstruídos podem ser projetados para simular tanto a epiderme quanto a pele de espessura total (FLATEN *et al.*, 2015; KÜCHLER; STRÜVER; FRIESS, 2013).

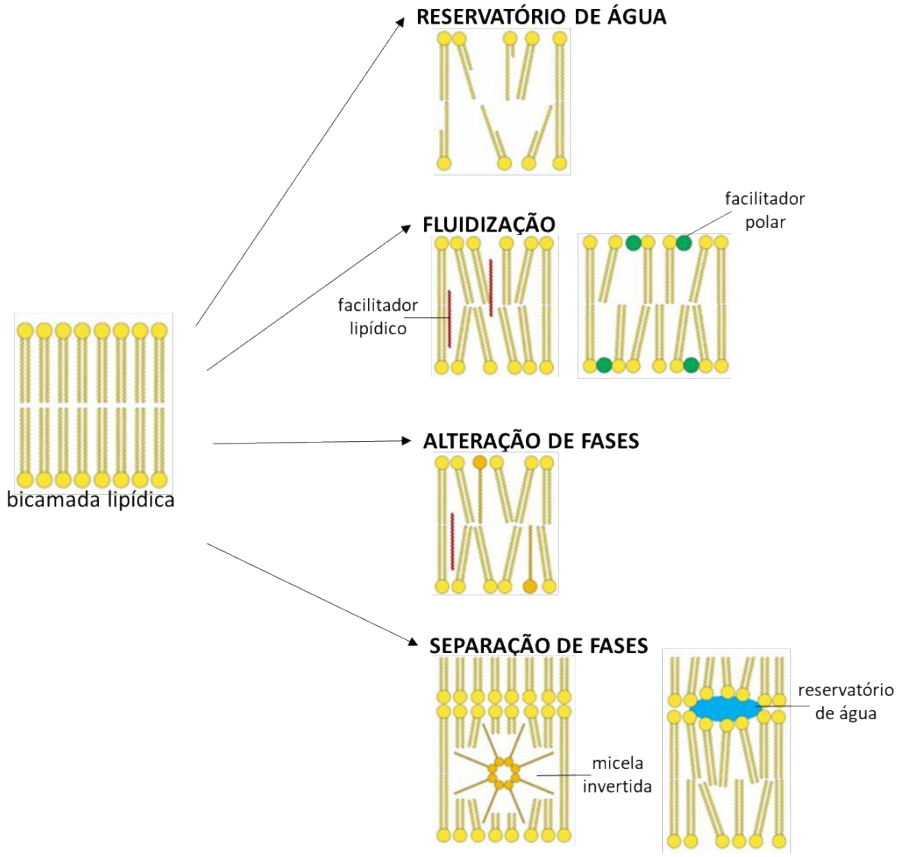
2.10. PROMOTORES DE ABSORÇÃO

Tendo em vista as propriedades de barreira do estrato córneo, promotores ou facilitadores de penetração/permeação podem ser incluídos na formulação. Estes compostos alteram o arranjo celular do tecido, facilitando a difusão do fármaco (PABLA; ZIA, 2007). Não devem possuir atividade farmacológica, ou seja, se ligar a receptores desencadeando respostas. Além disto, devem modificar a barreira da pele de forma "reversível". Uma vez que o promotor é removido, as ligações entre os lipídeos começam a se refazer, reconstituindo a estrutura original (KANG *et al.*, 2013). Ainda, devem ser atóxicos, não alergênicos, não irritantes e compatíveis com os componentes da formulação, inclusive o fármaco (HERMAN; HERMAN, 2015).

Os promotores de permeação podem agir rompendo a estrutura lipídica intercelular entre os corneócitos, fluidizando o estrato córneo; interagir com as proteínas do meio intercelular, melhorando a partição da molécula; agir nas conexões dos desmossomos entre os corneócitos ou alterar a atividade metabólica do tecido (Figura 6). Todos estes mecanismos podem modificar o arranjo estrutural do tecido e, consequentemente, diminuir as propriedades de barreira do estrato córneo (SILVA *et al.*, 2010; HERMAN; HERMAN, 2015; WILLIAMS; BARRY, 1991). O mecanismo preferencial dependerá das propriedades físico-químicas do fármaco e do promotor (GHAFOURIAN *et al.*, 2004).

A extração de lipídeos é realizada pelas formulações que contem álcoois como o etanol em concentrações de 40 a 80%. Esta extração promove a permeação de fármacos polares e apolares através de "poros" que são formados no tecido (DRAGICEVIC-CURIC; MAIBACH, 2015; LEVANG; ZHAO; SINGH, 1999). O etanol também é responsável pela fluidização dos lipídeos do estrato córneo em concentrações entre 20 a 60%, o que melhora a permeação de fármacos lipofílicos principalmente pela rota intercelular. A desorganização da camada lipídica também pode ocorrer devido a diferenças na hidratação do tecido (DRAGICEVIC-CURIC; MAIBACH, 2015).

Figura 6. Mecanismos de ação dos promotores de permeação.



Fonte: elaborado pelo autor.

Existem diversos tipos de promotores de permeação, desde sintéticos a naturais. Alguns representantes incluem sulfóxidos como o DMSO, Azone®, (primeiro composto desenvolvido para esta finalidade), pirrolidona, ácidos graxos, álcoois e glicol, tensoativos, ureia, óleos essenciais como os terpenos e fosfolipídios, dentre outros (KANG *et al.*, 2013; WILLIAMS; BARRY, 2012).

Neste trabalho, terpenos foram selecionados como agentes de permeação dada a origem natural que reduz chances de irritação ou problemas de biocompatibilidade (comuns para os promotores sintéticos). Estes compostos são metabólitos secundários e lipofílicos derivados do mevalonato e pirofosfato de isopentenilo. Aparecem na composição de óleos essenciais de vários produtos naturais (KAMATOU; VILJOEN, 2010).

De acordo com a quantidade de unidades de isoprenos que possuem, são classificados em hemiterpenos (C_5) monoterpenos (C_{10}), sesquiterpenos (C_{15}), diterpenos (C_{20}), sesterterpenos (C_{25}), triterpenos (C_{30}) e tetraterpenos (C_{40}), os quais

apresentam 1, 2, 3, 4, 5, 6 e 8 unidades de isopreno, respectivamente (KANG *et al.*, 2013; YAZAKI; ARIMURA; OHNISHI, 2017). Existe também uma outra classificação que se baseia na quantidade de anéis aromáticos (KANG *et al.*, 2013).

Para estudar o mecanismo de ação dos promotores de ação sobre o tecido, técnicas termoanalíticas (ex.: calorimetria exploratória diferencial – DSC) e espectroscópicas (espectroscopia no infravermelho por transformada de Fourier – FTIR) podem ser consideradas (SAPRA; JAIN; TIWARY, 2008). Comparando-se as endotermas e as exotermas da temperatura de transição (T_m), juntamente com as variações de entalpia (ΔH), é possível extrair informações a respeito do grau de desordem da camada lipídica e da desnaturação de proteínas (DRAGICEVIC-CURIC; MAIBACH, 2015). Termogramas de DSC de estrato córneo humano são caracterizados por três endotermas principais: 65°C, 75°C e 95°C. A primeira T_m indica fusão lipídica. A segunda endoterma tem relação com a quebra do complexo entre lipídeos e proteínas e a terceira podem ser associada à desnaturação das proteínas (LIM; LIU; CHAN, 2009).

As análises de FTIR apontam para mudanças conformacionais e moleculares nos lipídeos e proteínas do estrato córneo. Variações de alargamento/estreitamento bem como intensidade das bandas de FITR em relação a um controle (tecido não tratado) podem sugerir modificações químicas no estrato córneo. Na região entre 2.920 e 2.850 cm^{-1}, observam-se vibrações de C-H assimétricos e simétricos, respectivamente, as quais estão relacionadas à fluidização de lipídeos (LIM; LIU; CHAN, 2009). A quantidade de lipídeos está relacionada a intensidade e largura da banda. Alterações na intensidade e largura da banda sinalizam extração de lipídeos da pele (CHEN *et al.*, 2016). As vibrações C=O na região de 1.740 cm^{-1} referem-se as cabeças polares dos lipídeos. Variações na região entre 3.000 e 3.600 cm^{-1} sinalizam alterações no grau de hidratação. As bandas de 1.650 cm^{-1} e 1.550 cm^{-1} representam as amidas I e II das proteínas do estrato córneo (CHEN *et al.*, 2016; SAPRA; JAIN; TIWARY, 2008).

O principal mecanismo de ação dos terpenos é a alteração da fluidez do estrato córneo através da interação com os lipídeos intercelulares (HERMAN; HERMAN, 2015; JIANG *et al.*, 2017). O grau de desordem proporcionado por estes agentes parece estar relacionado com o tamanho da cadeia alquila, já que terpenos menores tem se mostrado mais efetivos que os sesquiterpenos (MOGHADAM *et al.*, 2013;

WILLIAMS; BARRY, 2012). A presença de anéis aromáticos mostrou um efeito negativo sobre a absorção de fármacos comparativamente aqueles terpenos com cadeias alquílicas longas (MOGHADAM *et al.*, 2013). Além disso, terpenos com baixo ponto de fusão mostraram maior ação reforçadora da permeação (JIANG *et al.*, 2017).

Em geral, terpenos com grupamentos polares ou oxigênio na sua estrutura tem se mostrado mais efetivos como promotores de permeação de moléculas hidrofílicas. Por outro lado, terpenos com hidrocarbonetos tem garantido um maior incremento da permeação de moléculas mais hidrofóbicas (GODWIN; MICHNIAK, 1999; KANG *et al.*, 2013; WILLIAMS; BARRY, 2012).

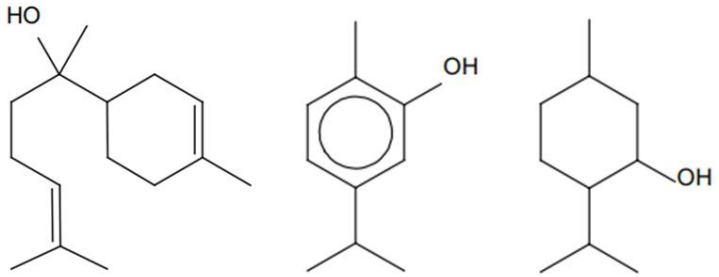
Kang *et al.* (2013) estabeleceram uma relação entre a presença de grupamentos químicos específicos no terpeno e a capacidade de promoção da permeação. Além de mencionar a importância do tamanho molecular, como já descrito, sugeriu a seguinte escala de permeação: éster > aldeído > óxido > hidrocarboneto > álcool > cetona > fenol > ácido. Assim, terpenos com grupamentos ésteres seriam os mais efetivos e os com grupamentos ácidos os menos efetivos.

Os terpenos são reconhecidamente seguros pela FDA (GRAS, *generally recognized as safe*), fato extremamente relevante já que muitos destes agentes podem penetrar a pele e alcançar a corrente sanguínea (SAPRA; JAIN; TIWARY, 2008; SONGKRO; RADES; BECKET, 2009a). Além de serem rapidamente metabolizados e excretados, (HERMAN; HERMAN, 2015), terpenos apresentam atividades farmacológicas específicas (WILLIAMS; BARRY, 2012), o que traz vantagens na utilização.

Nas formulações transdérmicas, geralmente emprega-se 5% (v/v) destes agentes, que é suficiente para a promoção da permeação de moléculas. O principal desafio é encontrar a concentração que promove alta permeação e, ao mesmo tempo, não resulte em toxicidade ou reações alérgicas/irritantes (KANG *et al.*, 2013).

Os terpenos selecionados para este trabalho foram o alfa-bisabolol, o carvacrol e o mentol (figura 7; tabela 1), os quais foram solubilizados em propilenoglicol, um cossolvente que age sinergicamente com os terpenos favorecendo a permeação de moléculas como a TST (ALAN ANDERSEN, 1999; BARRY; WILLIAMS, 1989; CHEN *et al.*, 2016; SONGKRO; RADES; BECKET, 2009a) .

Figura 7. Estruturas químicas dos terpenos α-bisabolol, carvacrol e mentol.



Fonte: Lim & Liu & Chan (2009).

Quadro 3. Propriedades físico-químicas dos terpenos α-bisabolol, carvacrol e mentol.

Terpeno	**Classe**	**Massa molar (g/mol)**	**Ponto de fusão (ºC)**	**Ponto de ebulição (ºC)**	**Log P**
α-bisabolol	Sesquiterpeno	222,37	Líquido	314,5	5,01 a 5,07
Carvacrol	Monoterpeno	150,22	3,5	237,7	3,28
Mentol	Monoterpeno	156,27	43	215,4	3,20

Fonte: Chen *et al.* (2016) e Kang *et al.* (2013).

Os terpenos com valores de log P mais elevados tendem a favorecer a permeação de moléculas mais lipofílicas como a TST, pois misturam-se com os lipídeos intercelulares e, desta forma, promovem maior fluidização e perturbação das propriedades de barreira do estrato córneo. O ponto de ebulição, por sua vez, relaciona-se de forma inversa com a taxa de permeação (CHEN *et al.*, 2016).

Abaixo estão descritas as características individuais dos terpenos utilizados nas formulações desenvolvidas neste trabalho.

2.10.1. Alfa-bisabolol

O α-bisabolol é um sesquiterpeno monocíclico insaturado. É extraído principalmente da *Matricaria chamomilla*, popularmente conhecida como camomila, mas também pode ser obtido sinteticamente. Foi isolado pela primeira vez em 1951, sendo mais estável na forma α-(-)-Bisabolol. Possui massa molar de 222,37 g/mol, densidade de 0,929 g/ml, ponto de ebulição de 315°C, sendo praticamente insolúvel em

água, porém, solúvel em álcoois como o propilenoglicol (BRUNKE; HAMMERSCHMIDT, 1985; KAMATOU; VILJOEN, 2010; KANG *et al.*, 2013; PERBELLINI *et al.*, 2004).

É caracterizado por um efeito anti-inflamatório e capacidade de diminuir o tempo de cicatrização (KADIR; BARRY, 1991; VILLEGAS *et al.*, 2001). Possui atividade antimicrobiana frente a patógenos como a *Candida albicans*, porém, menor que a apresentada pelo carvacrol (KAMATOU; VILJOEN, 2010). A literatura informa ainda que não há evidências de potencial teratogênico para o composto (ALAN ANDERSEN, 1999). Estudos apontam também a atividade inseticida e larvicida do α-bisabolol, atuando como repelente (FURTADO *et al.*, 2005; KAMATOU; VILJOEN, 2010).

Kim *et.al.* (2008) observaram que o α-bisabolol apresenta ação frente a hiperpigmentação cutânea, sendo útil para tratar patologias em que há aumento da produção de melanina como inflamações da pele, alergias de contato e dermatites ou ainda no melasma induzido por certas formulações (HASSUN; BAGATIN; VENTURA, 2008).

Com relação ao mecanismo de promoção da absorção, acredita-se que o α-bisabolol atue na fluidização dos lipídeos do estrato córneo, facilitando o particionamento de compostos na pele (CUI *et al.*, 2011; KADIR; BARRY, 1991). Cui *et.al.* (2011) destacam ainda que o α-bisabolol proporcionou a maior taxa de permeação para o cloridrato de propranolol dentre os promotores investigados (bornel e cânfora), além de prevenir a desidratação do estrato córneo e reduzir a irritação cutânea.

2.10.2. Carvacrol

O carvacrol é um monoterpeno fenólico, biossintetizado a partir do γ-terpineno passando pelo *p*-cimeno (CAN BASER, 2008). É líquido a temperatura ambiente, com densidade de 0,976 g/mL a 25°C, sendo insolúvel em água e muito solúvel em etanol, acetona e éter (YADAV; KAMBLE, 2009). Possui ação antimicrobiana, anticancerígena, antioxidante e analgésica (CAN BASER, 2008; SHARIFI-RAD *et al.*, 2018; SILVA *et al.*, 2010). É também indicado para problemas gastrointestinais,

redução dos níveis plasmáticos de colesterol e glicose, tratar dor no reumatismo e cicatrizante (BASER, 2002).

No estudo de permeação realizado por Songkro & Rades & Becket (2009), o carvacrol foi mais efetivo que o mentol no reforço da permeação do hormônio luteinizante. Este resultado foi atribuído as diferenças estruturais dos promotores de absorção (presença do fenol no carvacrol) (JEEVAN; VENKAT; KHAN, 1997; SONGKRO; BECKET; RADES, 2004). Quanto ao mecanismo de ação de promoção da absorção, acredita-se que o carvacrol haja modificando a barreira lipídica intercelular do tecido, com ganhos na absorção mais tardios (após um tempo de latência de 4 h) (LAOTHAWEERUNGSAWAT *et al.*, 2020).

2.10.3. Mentol

O mentol é um monoterpeno monocíclico, apresentando-se como um sólido cristalino à temperatura ambiente. Apresenta densidade de 0,890 Kg/dm³ a 25°C, ponto de fusão entre 41 e 44°C, é insolúvel em água e solúvel em formulações alcoólicas e propilenoglicol. É considerado seguro pelo FDA, com concentração aprovada para uso externo de até 16% (KAMATOU *et al.*, 2013).

É o óleo essencial das plantas *Mentha canadenses, Mentha x piperita* e *Mentha arvensis*, espécies semelhantes ao hortelã (GALEOTTI *et al.*, 2002; KAMATOU *et al.*, 2013). Pode ainda ser extraído e sintetizado do óleo de citronela, eucalipto e terebintina (GALEOTTI *et al.*, 2002). Produz ação refrescante, sendo cultivado no Japão há mais de 2000 anos atrás com finalidades medicinais (KAMATOU *et al.*, 2013). Foi isolado pela primeira vez em 1771 pelo botânico alemão Gambius (KAMATOU *et al.*, 2013; PATEL; ISHIUJI; YOSIPOVITCH, 2007).

Apresenta atividade antimicrobiana, anticancerígena, e anti-inflamatória, além de funcionar como repelente de insetos (KAMATOU *et al.*, 2013). Além disto, é utilizado para problemas estomacais, resfriado comum e demais complicações respiratórias leves bem como para dores musculares. Confere refrescância ao estimular os receptores de frio, inibindo o influxo de Ca^{2+} das membranas neuronais (ECCLES, 2011).

Kaplun-frischoff e Touitou (1997) relataram que o mentol foi capaz de aumentar a permeação da TST em oito vezes em relação a solução controle (TST livre) devido à formação de uma mistura eutética que diminuiu seu ponto de fusão de 153,7°C para 39,9°C. Além de aumentar a solubilidade da TST, o mentol parece afetar os espaços intercelulares (JEEVAN; VENKAT; KHAN, 1997).

Ainda, estudos sugerem que a ação do mentol envolve a extração de lipídeos do estrato córneo devido à interação com as cadeias apolares, o que foi confirmado pela redução da intensidade das bandas de FTIR relativas as vibrações de C-H. Estudos de FTIR também indicaram alterações nas vibrações de amidas, sugerindo interação com a queratina dos corneócitos (CHEN *et al.*, 2016).

REFERÊNCIAS

ABD, Eman *et al.* Skin models for the testing of transdermal drugs. **Clinical Pharmacology: Advances and Applications**, *[S. l.]*, v. 8, p. 163–176, 2016. Disponível em: https://doi.org/10.2147/CPAA.S64788

ACKERMANN, K. *et al.* The phenion® full-thickness skin model for percutaneous absorption testing. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 23, n. 2, p. 105–112, 2010. Disponível em: https://doi.org/10.1159/000265681

AINBINDER, Denize; TOUITOU, Elka. Testosterone ethosomes for enhanced transdermal delivery. **Drug delivery**, *[S. l.]*, v. 12, n. 5, p. 297–303, 2005.

ALAN ANDERSEN, F. Final report on the safety assessment of Bisabolol. **International Journal of Toxicology**, *[S. l.]*, v. 18, n. SUPPL. 3, p. 33–40, 1999. Disponível em: https://doi.org/10.1177/109158189901800305

ALBERTI, M. *et al.* Multi-chamber microfluidic platform for high-precision skin permeation testing. **Lab on a Chip**, *[S. l.]*, v. 17, n. 9, p. 1625–1634, 2017. Disponível em: https://doi.org/10.1039/c6lc01574c

ALJUFFALI, I. A.; LIN, C. F.; FANG, J. Y. Skin ablation by physical techniques for enhancing dermal/transdermal drug delivery. **Journal of Drug Delivery Science and Technology**, *[S. l.]*, v. 24, n. 3, p. 277–287, 2014. Disponível em: https://doi.org/10.1016/S1773-2247(14)50046-9

AMARU, Ricardo *et al.* HIPERTENSIÓN ARTERIAL PULMONAR EN PACIENTES CON ERITROCITOSIS PATOLÓGICAS. **Revista Médica La Paz**, *[S. l.]*, v. 25, n. 2, p. 27–35, 2019.

ARAYA, Hiroshi; TOMITA, Mikio; HAYASHI, Masahiro. The novel formulation design of O/W microemulsion for improving the gastrointestinal absorption of poorly water soluble compounds. **International Journal of Pharmaceutics**, *[S. l.]*, v. 305, n. 1–2, p. 61–74, 2005. Disponível em: https://doi.org/10.1016/j.ijpharm.2005.08.022

ARVER, S. *et al.* Long-term efficacy and safety of a permeation-enhanced testosterone transdermal system in hypogonadal men. **Clinical Endocrinology**, Reproductive Medical Centre, Karolinska Hospital, Stockholm, Sweden, v. 47, n. 6, p. 727–737, 1997. Disponível em: https://doi.org/10.1046/j.1365-2265.1997.3071113.x

ASHCROFT, Gillian S.; MILLS, Stuart J. Androgen receptor–mediated inhibition of cutaneous wound healing. **Journal of Clinical Investigation**, *[S. l.]*, v. 110, n. 5, p. 615–624, 2002. Disponível em: https://doi.org/10.1172/JCI15704

ATRUX-TALLAU, Nicolas *et al.* Qualitative and quantitative comparison of heat separated epidermis and dermatomed skin in percutaneous absorption studies. **Archives of Dermatological Research**, *[S. l.]*, v. 299, n. 10, p. 507–511, 2007. Disponível em: https://doi.org/10.1007/s00403-007-0789-y

AULTON, M. E.; TAYLOR, K. M. .. **Aulton: Delineamento de Formas Farmacêuticas**. 4. ed ed. Rio de Janeiro: Elsevier, 2016. *E-book*.

AZAD, Nasrin *et al.* Testosterone Treatment Enhances Regional Brain Perfusion in Hypogonadal Men. **The Journal of Clinical Endocrinology & Metabolism**, *[S. l.]*, v. 88, n. 7, p. 3064–3068, 2003. Disponível em: https://doi.org/10.1210/jc.2002-020632

BAERT, B. *et al.* Increase of the transdermal penetration of testosterone by miconazole nitrate. **Archives of Pharmacal Research**, *[S. l.]*, v. 35, n. 12, p. 2163–2170, 2012. Disponível em: https://doi.org/10.1007/s12272-012-1214-4

BAIN, Jerald. The many faces of testosterone. **Clinical Interventions in Aging**, *[S. l.]*, v. Volume 2, p. 567–576, 2008. Disponível em: https://doi.org/10.2147/CIA.S1417

BAKONYI, Mónika *et al.* Stratum corneum lipid liposomes for investigating skin penetration enhancer effects. **RSC Advances**, *[S. l.]*, v. 8, n. 48, p. 27464–27469, 2018.

Disponível em: https://doi.org/10.1039/C8RA04129F

BARBER, E. D. *et al.* A comparative study of the rates of in vitro percutaneous absorption of eight chemicals using rat and human skin. **Fundamental and Applied Toxicology**, *[S. l.]*, v. 19, n. 4, p. 493–497, 1992. Disponível em: https://doi.org/10.1016/0272-0590(92)90086-W

BARBERO, Ana M.; FRASCH, H. Frederick. Pig and guinea pig skin as surrogates for human in vitro penetration studies: A quantitative review. **Toxicology in Vitro**, *[S. l.]*, v. 23, n. 1, p. 1–13, 2009. Disponível em: https://doi.org/10.1016/j.tiv.2008.10.008

BARBERO, Ana M.; FRASCH, H. Frederick. Effect of Frozen Human Epidermis Storage Duration and Cryoprotectant on Barrier Function Using Two Model Compounds. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 29, n. 1, p. 31–40, 2016. Disponível em: https://doi.org/10.1159/000441038

BARRY, B. W. Mode of action of penetration enhancers in human skin. **Journal of Controlled Release**, *[S. l.]*, v. 6, n. 1, p. 85–97, 1987. Disponível em: https://doi.org/10.1016/0168-3659(87)90066-6

BARRY, B. W.; WILLIAMS, A. C. Human skin penetration enhancement: the synergy of propylene glycol with terpenes. **16th Iniernational Symposium, Controhed Release of Bioactioe Materials,** *[S. l.]*, v. 16, p. 33–34, 1989.

BARTOSOVA, L.; BAJGAR, J. Transdermal Drug Delivery In Vitro Using Diffusion Cells. **Current Medicinal Chemistry**, *[S. l.]*, v. 19, n. 27, p. 4671–4677, 2012. Disponível em: https://doi.org/10.2174/092986712803306358

BASARIA, Shehzad; DOBS, Adrian S. Hypogonadism and androgen replacement therapy in elderly men. **The American journal of medicine**, *[S. l.]*, v. 110, n. 7, p. 563–572, 2001.

BASER, KHC. The Turkish Origanum Species. *In*: KINTZIOS, Spiridon E. (org.). **Oregano, The Genera Origanum and Lippia**. *[S. l.]*: CRC Press, 2002. p. 109–126. *E-book*. Disponível em: https://doi.org/10.1201/b12591

BAULIEU, E. E. Neurosteroids: of the nervous system, by the nervous system, for the nervous system. **Recent progress in hormone research**, *[S. l.]*, v. 52, p. 1–32, 1997. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/9238846

BAUMANN, G. Growth hormone binding protein. The soluble growth hormone receptor. **Minerva endocrinologica**, *[S. l.]*, v. 27, n. 4, p. 265–276, 2002. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/12511849

BEACH, FA. **Hormones and behavior**. New York: Hoeber, 1948. *E-book*.

BECKER, JB; BREEDLOVE, JM; CREWS, D. **Behavioral endocrinology**. *[S. l.]*: MIT Press, 1992. *E-book*.

BEDIMO, Roger *et al.* Osteoporotic fracture risk associated with cumulative exposure to tenofovir and other antiretroviral agents. **AIDS**, *[S. l.]*, v. 26, n. 7, p. 825–831, 2012. Disponível em: https://doi.org/10.1097/QAD.0b013e32835192ae

BENFELDT, Eva *et al.* Bioequivalence of Topical Formulations in Humans: Evaluation by Dermal Microdialysis Sampling and the Dermatopharmacokinetic Method. **Journal of Investigative Dermatology**, *[S. l.]*, v. 127, n. 1, p. 170–178, 2007. Disponível em: https://doi.org/10.1038/sj.jid.5700495

BENNION, Brian J. *et al.* Predicting a Drug's Membrane Permeability: A Computational Model Validated With in Vitro Permeability Assay Data. **The Journal of Physical Chemistry B**, *[S. l.]*, v. 121, n. 20, p. 5228–5237, 2017. Disponível em: https://doi.org/10.1021/acs.jpcb.7b02914

BERNARD, Philippe; SCIOR, Thomas; DO, Quoc Tuan. Modulating testosterone pathway: a new strategy to tackle male skin aging? **Clinical Interventions in Aging**, *[S. l.]*, p. 351, 2012. Disponível em: https://doi.org/10.2147/CIA.S34034

BETTINI, Ezio *et al.* Estrogen Receptor in Rat Brain Presence in the Hippocampal Formation. **Neuroendocrinology**, *[S. l.]*, v. 56, n. 4, p. 502–508, 1992. Disponível em: https://doi.org/10.1159/000126267

BHASIN, S. *et al.* Testosterone therapy in men with androgen deficiency syndromes: an Endocrine Society clinical practice guideline. **J Clin Endocrinol Task force, endocrine society**, *[S. l.]*, n. 95, p. 536–559, 2010.

BHASIN, Shalender *et al.* Managing the Risks of Prostate Disease During Testosterone Replacement Therapy in Older Men: Recommendations for a Standardized Monitoring Plan. **Journal of Andrology**, *[S. l.]*, v. 24, n. 3, p. 299–311, 2003. Disponível em: https://doi.org/10.1002/j.1939-4640.2003.tb02676.x

BINKS, Bernard P. *et al.* Membrane Permeation of Testosterone from Either Solutions, Particle Dispersions, or Particle-Stabilized Emulsions. **Langmuir**, *[S. l.]*, v. 28, n. 5, p. 2510–2522, 2012. Disponível em: https://doi.org/10.1021/la204755m

BIZZARRO, A. *et al.* Influence of Testosterone Therapy on Clinical and Immunological Features of Autoimmune Diseases Associated with Klinefelter's Syndrome. **The Journal of Clinical Endocrinology & Metabolism**, *[S. l.]*, v. 64, n. 1, p. 32–36, 1987. Disponível em: https://doi.org/10.1210/jcem-64-1-32

BLYTHE, Linda Lou; GANNON, James R.; CRAIG, A. Morrie. **Care of the racing greyhound**. *[S. l.]*: American Greyhound Council, 1994. *E-book*.

BOER, Magdalena *et al.* Structural and biophysical characteristics of human skin in maintaining proper epidermal barrier function. **Advances in Dermatology and Allergology**, *[S. l.]*, v. 1, p. 1–5, 2016. Disponível em: https://doi.org/10.5114/pdia.2015.48037

BONINA, F. P. *et al.* Vehicle effects on in vitro skin permeation of and stratum corneum affinity for model drugs caffeine and testosterone. **International Journal of Pharmaceutics**, *[S. l.]*, v. 100, n. 1–3, p. 41–47, 1993. Disponível em: https://doi.org/10.1016/0378-5173(93)90073-O

BORST, Stephen E. *et al.* Cardiovascular risks and elevation of serum DHT vary by route of testosterone administration: a systematic review and meta-analysis. **BMC Medicine**, *[S. l.]*, v. 12, n. 1, p. 211, 2014. Disponível em: https://doi.org/10.1186/s12916-014-0211-5

BOUWSTRA, J. Structure of the skin barrier and its modulation by vesicular formulations. **Progress in Lipid Research**, *[S. l.]*, v. 42, n. 1, p. 1–36, 2003. Disponível em: https://doi.org/10.1016/S0163-7827(02)00028-0

BRAIN, K.; WALTERS, K.; WATKINSON, A. Methods for Studying Percutaneous Absorption, Drugs and the Pharmaceutical Sciences. *In*: **Percutaneous Absorption Drugs, Cosmetics, Mechanisms, Methods**. *[S. l.: s. n.]*. p. 197–269. *E-book*.

BRAIN, P.; HAUG, M. Hormonal and neurochemical correlates of various forms of animal "aggression". **Psychoneuroendocrinology**, *[S. l.]*, v. 17, n. 6, p. 537–551, 1992. Disponível em: https://doi.org/10.1016/0306-4530(92)90014-X

BREMNER, William J.; VITIELLO, Michael V.; PRINZ, Patricia N. Loss of Circadian Rhythmicity in Blood Testosterone Levels with Aging in Normal Men. **The Journal of Clinical Endocrinology & Metabolism**, *[S. l.]*, v. 56, n. 6, p. 1278–1281, 1983. Disponível em: https://doi.org/10.1210/jcem-56-6-1278

BRIDGES, N. A. *et al.* The relationship between endogenous testosterone and gonadotrophin secretion. **Clinical Endocrinology**, *[S. l.]*, v. 38, n. 4, p. 373–378, 1993. Disponível em: https://doi.org/10.1111/j.1365-2265.1993.tb00517.x

SEBACEOUS GLAND. *In*: BRITANNICA, The Editors of Encyclopaedia. **Encyclopedia Britannica**., *[s. d.]*. p. https://www.britannica.com/science/sebaceous-gland.

BRONAUGH, Robert L.; STEWART, Raymond F. Methods for in vitro percutaneous absorption studies IV: The flow-through diffusion cell. **Journal of pharmaceutical sciences**, *[S. l.]*, v. 74, n. 1, p. 64–67, 1985.

BROWN, M. B. *et al.* Dermal and transdermal drug delivery systems: Current and future prospects. **Drug Delivery: Journal of Delivery and Targeting of Therapeutic Agents**, King's College London, Pharmaceutical Sciences Research Division, London, United Kingdom, v. 13, n. 3, p. 175–187, 2006. Disponível em: https://doi.org/10.1080/10717540500455975

BROWN, Marybeth. Skeletal muscle and bone: effect of sex steroids and aging. **Advances in Physiology Education**, *[S. l.]*, v. 32, n. 2, p. 120–126, 2008. Disponível em: https://doi.org/10.1152/advan.90111.2008

BRUNKE, E. J.; HAMMERSCHMIDT, F. J. Constituents of the Essential Oil of Salvia Stenophylla — First Identification of (+)-Epi-α-Bisabolol in Nature. *In*: **Essential Oils and Aromatic Plants**. Dordrecht: Springer Netherlands, 1985. p. 145–150. *E-book*. Disponível em: https://doi.org/10.1007/978-94-009-5137-2_13

BUTENANDT, Adolf; HANISCH, Günter. Über die Umwandlung des Dehydro-androsterons in Δ 4 -Androsten-ol-(17)-0n-(3) (Testosteron); ein Weg zur Darstellung des Testosterons aus Cholesterin (Vorläuf. Mitteil.). **Berichte der deutschen chemischen Gesellschaft (A and B Series)**, *[S. l.]*, v. 68, n. 9, p. 1859–1862, 1935. Disponível em: https://doi.org/10.1002/cber.19350680937

CALOF, O. M. *et al.* Adverse Events Associated With Testosterone Replacement in Middle-Aged and Older Men: A Meta-Analysis of Randomized, Placebo-Controlled Trials. **The Journals of Gerontology Series A: Biological Sciences and Medical Sciences**, *[S. l.]*, v. 60, n. 11, p. 1451–1457, 2005. Disponível em: https://doi.org/10.1093/gerona/60.11.1451

CAN BASER, K. Biological and Pharmacological Activities of Carvacrol and Carvacrol Bearing Essential Oils. **Current Pharmaceutical Design**, *[S. l.]*, v. 14, n. 29, p. 3106–3119, 2008. Disponível em: https://doi.org/10.2174/138161208786404227

CAON, Thiago *et al.* Novel perspectives in the tuberculosis treatment: Administration of isoniazid through the skin. **International Journal of Pharmaceutics**, *[S. l.]*, v. 494, n. 1, p. 463–470, 2015. Disponível em: https://doi.org/10.1016/j.ijpharm.2015.08.067

CAPT, A. *et al.* Comparison of the human skin grafted onto nude mouse model with in vivo and in vitro models in the prediction of percutaneous penetration of three lipophilic pesticides. **Regulatory Toxicology and Pharmacology**, *[S. l.]*, v. 47, n. 3, p. 274–287, 2007. Disponível em: https://doi.org/10.1016/j.yrtph.2006.11.008

CARRER, Victor *et al.* Effect of propylene glycol on the skin penetration of drugs. **Archives of dermatological research**, *[S. l.]*, v. 312, n. 5, p. 337–352, 2020.

CAUSSIN, Julia *et al.* Lipid organization in human and porcine stratum corneum differs widely, while lipid mixtures with porcine ceramides model human stratum corneum lipid organization very closely. **Biochimica et Biophysica Acta (BBA) - Biomembranes**, *[S. l.]*, v. 1778, n. 6, p. 1472–1482, 2008. Disponível em: https://doi.org/10.1016/j.bbamem.2008.03.003

ČEPONIS, J. *et al.* Testosterone therapy: Transdermal androgens. *In*: **Testosterone: From Basic to Clinical Aspects**. *[S. l.: s. n.]*. p. 225–235. *E-book*. Disponível em: https://doi.org/10.1007/978-3-319-46086-4_11

CESCHEL, GianCarlo *et al.* Solubility and Transdermal Permeation Properties of a Dehydroepiandrosterone Cyclodextrin Complex from Hydrophilic and Lipophilic Vehicles. **Drug Delivery**, *[S. l.]*, v. 12, n. 5, p. 275–280, 2005. Disponível em: https://doi.org/10.1080/10717540500176563

CHEMES, HE. Leydig cell development in humans. *In*: PAYNE, AH; HARDY, MP;

RUSSELL, LD (org.). **The Leydig cell**. Vienna: Cache River Press, 1996. p. 176–202. *E-book*.

CHEN, Jun *et al.* Natural Terpenes as Penetration Enhancers for Transdermal Drug Delivery. **Molecules**, *[S. l.]*, v. 21, n. 12, p. 1709, 2016. Disponível em: https://doi.org/10.3390/molecules21121709

CHEN, WenChieh; THIBOUTOT, Diane; ZOUBOULIS, Christos C. Cutaneous Androgen Metabolism: Basic Research and Clinical Perspectives. **Journal of Investigative Dermatology**, *[S. l.]*, v. 119, n. 5, p. 992–1007, 2002. Disponível em: https://doi.org/10.1046/j.1523-1747.2002.00613.x

CHOUDHRY, R. *et al.* Localization of androgen receptors in human skin by immunohistochemistry: implications for the hormonal regulation of hair growth, sebaceous glands and sweat glands. **Journal of Endocrinology**, *[S. l.]*, v. 133, n. 3, p. 467- NP, 1992. Disponível em: https://doi.org/10.1677/joe.0.1330467

CIGARRÁN, Secundino *et al.* Endogenous testosterone, muscle strength, and fat-free mass in men with chronic kidney disease. **Journal of Renal Nutrition**, *[S. l.]*, v. 23, n. 5, 2013. Disponível em: https://doi.org/10.1053/j.jrn.2012.08.007

CILURZO, Francesco *et al.* Design of in vitro skin permeation studies according to the EMA guideline on quality of transdermal patches. **European Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 125, p. 86–92, 2018. Disponível em: https://doi.org/10.1016/j.ejps.2018.09.014

CLOWES, H. M.; SCOTT, R. C.; HEYLINGS, J. R. Skin absorption: flow-through or static diffusion cells. **Toxicology in vitro**, *[S. l.]*, v. 8, n. 4, p. 827–830, 1994.

COFFEY, DS. Androgen action and the sex accessory tissues. *In*: KNOBI, l E.; NEILL, J. (org.). **The physiology of reproduction**. New York: Raven Press, 1988. p. 1081–1119. *E-book*.

COTTE, Marine *et al.* Synchrotron FT-IR microscopic study of chemical enhancers in transdermal drug delivery: example of fatty acids. **Journal of Controlled Release**, *[S. l.]*, v. 97, n. 2, p. 269–281, 2004. Disponível em: https://doi.org/10.1016/j.jconrel.2004.03.014

COUTO, Ana *et al.* Dermic diffusion and stratum corneum: A state of the art review of

mathematical models. **Journal of Controlled Release**, *[S. l.]*, v. 177, p. 74–83, 2014. Disponível em: https://doi.org/10.1016/j.jconrel.2013.12.005

COVIELLO, Andrea D. *et al.* Effects of Graded Doses of Testosterone on Erythropoiesis in Healthy Young and Older Men. **The Journal of Clinical Endocrinology & Metabolism**, *[S. l.]*, v. 93, n. 3, p. 914–919, 2008. Disponível em: https://doi.org/10.1210/jc.2007-1692

CROSS, Sheree E. *et al.* Determination of the effect of lipophilicity on the in vitro permeability and tissue reservoir characteristics of topically applied solutes in human skin layers. **Journal of Investigative Dermatology**, *[S. l.]*, v. 120, n. 5, p. 759–764, 2003. Disponível em: https://doi.org/10.1046/j.1523-1747.2003.12131.x

CUI, Ying *et al.* Enhancement and mechanism of transdermal absorption of terpene-induced propranolol hydrochloride. **Archives of Pharmacal Research**, *[S. l.]*, v. 34, n. 9, p. 1477–1485, 2011. Disponível em: https://doi.org/10.1007/s12272-011-0909-2

CUNNINGHAM, Glenn R. Testosterone Replacement With Transdermal Therapeutic Systems. **JAMA**, *[S. l.]*, v. 261, n. 17, p. 2525, 1989. Disponível em: https://doi.org/10.1001/jama.1989.03420170069032

CUTOLO, Maurizio. SEX HORMONE ADJUVANT THERAPY IN RHEUMATOID ARTHRITIS. **Rheumatic Disease Clinics of North America**, *[S. l.]*, v. 26, n. 4, p. 881–895, 2000. Disponível em: https://doi.org/10.1016/S0889-857X(05)70174-5

DA SILVA, José Alexsandro *et al.* Administração cutânea de fármacos: Desafios e estratégias para o desenvolvimento de formulações transdérmicas. **Revista de Ciencias Farmaceuticas Basica e Aplicada**, *[S. l.]*, v. 31, n. 3, 2010.

DAVID, K. *et al.* Über krystallinisches männliches Hormon aus Hoden (Testosteron), wirksamer als aus Harn oder aus Cholesterin bereitetes Androsteron. **Hoppe-Seyler´s Zeitschrift für physiologische Chemie**, *[S. l.]*, v. 233, n. 5–6, p. 281–283, 1935. Disponível em: https://doi.org/10.1515/bchm2.1935.233.5-6.281

DAVIES, D. J.; WARD, R. J.; HEYLINGS, J. R. Multi-species assessment of electrical resistance as a skin integrity marker for in vitro percutaneous absorption studies. **Toxicology in Vitro**, *[S. l.]*, v. 18, n. 3, p. 351–358, 2004. Disponível em: https://doi.org/10.1016/j.tiv.2003.10.004

DOBS, A. S. *et al.* A Novel Testosterone 2% Gel for the Treatment of Hypogonadal Males. **Journal of Andrology**, *[S. l.]*, v. 33, n. 4, p. 601–607, 2012. Disponível em: https://doi.org/10.2164/jandrol.111.014308

DOHLE, G. R. *et al.* **Guidelines on male hypogonadism**. *[S. l.]*: Eur. Assoc. Urol., 2012.

DOHLE, G. R. *et al.* EAU Guidelines on Male Hypogonadism. **Europian Association of Urology**, *[S. l.]*, 2018.

DOMINGUEZ, S.; MACKERT, G. A.; DOBKE, M. .. Nanotechnology to enhance transdermal delivery of hydrophilic humectants for improved skin care: a model for therapeutic applications. *In*: ANDRONESCU, E.; GRUMEZESCU, A. M. (org.). **Nanostructures for Drug Delivery**. *[S. l.]*: Elsevier, 2017. p. 919–939. *E-book*.

DRABIK, A.; BODZOŃ-KUŁAKOWSKA, A.; SILBERRING, J. 7 - Gel Electrophoresis. *In*: CIBOROWSKI, P.; SILBERRING, J. B. T. Proteomic Profiling and Analytical Chemistry (Second Edition) (org.). Boston: Elsevier, 2016. p. 115–143. *E-book*. Disponível em: https://doi.org/https://doi.org/10.1016/B978-0-444-63688-1.00007-0

DRAGICEVIC-CURIC, Nina; MAIBACH, Howard I. **Percutaneous Penetration Enhancers Chemical Methods in Penetration Enhancement**. Berlin, Heidelberg: Springer Berlin Heidelberg, 2015. *E-book*. Disponível em: https://doi.org/10.1007/978-3-662-45013-0

ECCLES, R. Menthol and Related Cooling Compounds. **Journal of Pharmacy and Pharmacology**, *[S. l.]*, v. 46, n. 8, p. 618–630, 2011. Disponível em: https://doi.org/10.1111/j.2042-7158.1994.tb03871.x

ERNESTI, A. M.; SWIDEREK, M.; GAY, R. Absorption and metabolism of topically applied testosterone in an organotypic skin culture. **Skin Pharmacology**, *[S. l.]*, v. 5, n. 3, p. 146–153, 1992. Disponível em: https://doi.org/10.1159/000211031

ESCOBAR-CHAVEZ, Jose Juan *et al.* The Tape-Stripping Technique as a Method for Drug Quantification in Skin. **Journal of Pharmacy & Pharmaceutical Sciences**, *[S. l.]*, v. 11, n. 1, p. 104, 2008. Disponível em: https://doi.org/10.18433/J3201Z

ESPOSTO BIONDO, Nicole *et al.* How to define the experimental conditions of skin

permeation assays for drugs presenting biopharmaceutical limitations? The experience with testosterone. **International Journal of Pharmaceutics**, *[S. l.]*, v. 607, p. 120987, 2021. Disponível em: https://doi.org/10.1016/j.ijpharm.2021.120987

ÉVORA, Ana S. *et al.* Corneocytes: Relationship between Structural and Biomechanical Properties. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 34, n. 3, p. 146–161, 2021. Disponível em: https://doi.org/10.1159/000513054

FABIAN, Eric *et al.* A protocol to determine dermal absorption of xenobiotica through human skin in vitro. **Archives of Toxicology**, *[S. l.]*, v. 91, n. 3, p. 1497–1511, 2017. Disponível em: https://doi.org/10.1007/s00204-016-1782-z

FAHMY, F. S. *et al.* Skin graft storage and keratinocyte viability. **British Journal of Plastic Surgery**, *[S. l.]*, v. 46, n. 4, p. 292–295, 1993. Disponível em: https://doi.org/10.1016/0007-1226(93)90005-V

FANG, Jia-You *et al.* In vitro and in vivo evaluations of the efficacy and safety of skin permeation enhancers using flurbiprofen as a model drug. **International Journal of Pharmaceutics**, *[S. l.]*, v. 255, n. 1–2, p. 153–166, 2003. Disponível em: https://doi.org/10.1016/S0378-5173(03)00086-3

FERNÁNDEZ-BALSELLS, M. Mercè *et al.* Adverse Effects of Testosterone Therapy in Adult Men: A Systematic Review and Meta-Analysis. **The Journal of Clinical Endocrinology & Metabolism**, *[S. l.]*, v. 95, n. 6, p. 2560–2575, 2010. Disponível em: https://doi.org/10.1210/jc.2009-2575

FINNIN, Barrie; WALTERS, Kenneth A.; FRANZ, Thomas J. In Vitro Skin Permeation Methodology. *In*: BENSON, A.E., Heather; WATKINSON, Adam C. (org.). **Transdermal and Topical Drug Delivery: Principles and Practice**. First Edit ed. *[S. l.]*: John Wiley & Sons, Inc, 2012. p. 85–108. *E-book*.

FLATEN, Gøril Eide *et al.* In vitro skin models as a tool in optimization of drug formulation. **European Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 75, p. 10–24, 2015. Disponível em: https://doi.org/10.1016/j.ejps.2015.02.018

FLYNN, G. L.; YALKOWSKY, S. H.; ROSEMAN, T. J. Mass transport Phenomena and Models: Theoretical Concepts. **Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 63, n. 4, p. 479–510, 1974. Disponível em: https://doi.org/10.1002/jps.2600630403

FOX, Lizelle T. *et al.* Transdermal Drug Delivery Enhancement by Compounds of Natural Origin. **Molecules**, *[S. l.]*, v. 16, n. 12, p. 10507–10540, 2011. Disponível em: https://doi.org/10.3390/molecules161210507

FURTADO, Roselayne F. *et al.* Atividade larvicida de óleos essenciais contra Aedes aegypti L. (Diptera: Culicidae). **Neotropical Entomology**, *[S. l.]*, v. 34, n. 5, p. 843–847, 2005. Disponível em: https://doi.org/10.1590/S1519-566X2005000500018

GALEOTTI, Nicoletta *et al.* Menthol: a natural analgesic compound. **Neuroscience Letters**, *[S. l.]*, v. 322, n. 3, p. 145–148, 2002. Disponível em: https://doi.org/10.1016/S0304-3940(01)02527-7

GARIDEL, Patrick. Mid-FTIR-Microspectroscopy of stratum corneum single cells and stratum corneum tissue. **Physical Chemistry Chemical Physics**, *[S. l.]*, v. 4, n. 22, p. 5671–5677, 2002. Disponível em: https://doi.org/10.1039/b207478h

GHAFOURIAN, Taravat *et al.* The effect of penetration enhancers on drug delivery through skin: a QSAR study. **Journal of Controlled Release**, *[S. l.]*, v. 99, n. 1, p. 113–125, 2004. Disponível em: https://doi.org/10.1016/j.jconrel.2004.06.010

GIRARDEAU-HUBERT, Sarah *et al.* Reconstructed Skin Models Revealed Unexpected Differences in Epidermal African and Caucasian Skin. **Scientific Reports**, *[S. l.]*, v. 9, n. 1, p. 7456, 2019. Disponível em: https://doi.org/10.1038/s41598-019-43128-3

GODIN, B.; TOUITOU, E. Transdermal skin delivery: Predictions for humans from in vivo, ex vivo and animal models. **Advanced Drug Delivery Reviews**, Department of Pharmaceutics, School of Pharmacy, Faculty of Medicine, Jerusalem, 91120, Israel, v. 59, n. 11, p. 1152–1161, 2007. Disponível em: https://doi.org/10.1016/j.addr.2007.07.004

GODWIN, D. A.; MICHNIAK, B. B. Influence of Drug Lipophilicity on Terpenes as Transdermal Penetration Enhancers. **Drug Development and Industrial Pharmacy**, *[S. l.]*, v. 25, n. 8, p. 905–915, 1999. Disponível em: https://doi.org/10.1081/DDC-100102251

GOLDEN, Guia M. *et al.* Lipid Thermotropic Transitions in Human Stratum Corneum. **Journal of Investigative Dermatology**, *[S. l.]*, v. 86, n. 3, p. 255–259, 1986.

Disponível em: https://doi.org/10.1111/1523-1747.ep12285373

GONZALEZ, J. P.; BROGDEN, R. N. Naltrexone: A Review of its Pharmacodynamic and Pharmacokinetic Properties and Therapeutic Efficacy in the Management of Opioid Dependence. **Drugs**, *[S. l.]*, v. 35, n. 3, p. 192–213, 1988. Disponível em: https://doi.org/10.2165/00003495-198835030-00002

GRAY, G. M.; YARDLEY, H. J. Lipid compositions of cells isolated from pig, human, and rat epidermis. **Journal of lipid research**, *[S. l.]*, v. 16, n. 6, p. 434–440, 1975. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/1194786

GUILLARD, Emmanuelle Corbe *et al.* Molecular interactions of penetration enhancers within ceramides organization: A FTIR approach. **European Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 36, n. 2–3, p. 192–199, 2009. Disponível em: https://doi.org/10.1016/j.ejps.2008.10.010

GUTH, K. *et al.* Suitability of skin integrity tests for dermal absorption studies in vitro. **Toxicology in Vitro**, *[S. l.]*, v. 29, n. 1, p. 113–123, 2015. Disponível em: https://doi.org/10.1016/j.tiv.2014.09.007

GÜVENÇ, Mehmet *et al.* Effects of thymol and carvacrol on sperm quality and oxidant/antioxidant balance in rats. **Archives of Physiology and Biochemistry**, *[S. l.]*, v. 125, n. 5, p. 396–403, 2019. Disponível em: https://doi.org/10.1080/13813455.2018.1476979

GYSLER, A. *et al.* Skin penetration and metabolism of topical glucocorticoids in reconstructed epidermis and in excised human skin. **Pharmaceutical research**, *[S. l.]*, v. 16, n. 9, p. 1386–1391, 1999. Disponível em: https://doi.org/10.1023/a:1018946924585

HADDAD, Rudy M. *et al.* Testosterone and Cardiovascular Risk in Men: A Systematic Review and Meta-analysis of Randomized Placebo-Controlled Trials. **Mayo Clinic Proceedings**, *[S. l.]*, v. 82, n. 1, p. 29–39, 2007. Disponível em: https://doi.org/10.4065/82.1.29

HADGRAFT, Jonathan. Skin deep. **European Journal of Pharmaceutics and Biopharmaceutics**, *[S. l.]*, v. 58, n. 2, p. 291–299, 2004. Disponível em: https://doi.org/10.1016/j.ejpb.2004.03.002

HADGRAFT, Jonathan; LANE, Majella E. Transdermal delivery of testosterone. **European Journal of Pharmaceutics and Biopharmaceutics**, *[S. l.]*, v. 92, p. 42–48, 2015. Disponível em: https://doi.org/10.1016/j.ejpb.2015.02.015

HAQ, Anika *et al.* Membrane properties for permeability testing: Skin versus synthetic membranes. **International Journal of Pharmaceutics**, *[S. l.]*, v. 539, n. 1–2, p. 58–64, 2018. Disponível em: https://doi.org/10.1016/j.ijpharm.2018.01.029

HAQ, Anika; MICHNIAK-KOHN, Bozena. Effects of solvents and penetration enhancers on transdermal delivery of thymoquinone: Permeability and skin deposition study. **Drug delivery**, *[S. l.]*, v. 25, n. 1, p. 1943–1949, 2018.

HASSUN, Karime M.; BAGATIN, Ediléia; VENTURA, Karin Ferreira. Melasma: [revisão]. **RBM rev. bras. med**, *[S. l.]*, v. 65, p. 11–16, 2008.

HELLMAN, LEON; ROSENFELD, R. S. Metabolism of Testosterone-1,2- 3 H in Man. Distribution of the Major 17-Ketosteroid Metabolites in Plasma: Relation to Thyroid States. **The Journal of Clinical Endocrinology & Metabolism**, *[S. l.]*, v. 38, n. 3, p. 424–435, 1974. Disponível em: https://doi.org/10.1210/jcem-38-3-424

HERBST, Karen L.; BHASIN, Shalender. Testosterone action on skeletal muscle. **Current Opinion in Clinical Nutrition and Metabolic Care**, *[S. l.]*, v. 7, n. 3, p. 271–277, 2004. Disponível em: https://doi.org/10.1097/00075197-200405000-00006

HERMAN, Anna; HERMAN, Andrzej P. Essential oils and their constituents as skin penetration enhancer for transdermal drug delivery: a review. **Journal of Pharmacy and Pharmacology**, *[S. l.]*, v. 67, n. 4, p. 473–485, 2015. Disponível em: https://doi.org/10.1111/jphp.12334

HEWITT, N. J. *et al.* Measurement of the penetration of 56 cosmetic relevant chemicals into and through human skin using a standardized protocol. **Journal of Applied Toxicology**, *[S. l.]*, v. 40, n. 3, p. 403–415, 2020. Disponível em: https://doi.org/10.1002/jat.3913

HEYLINGS, J. R.; DAVIES, D. J.; BURTON, R. Dermal absorption of testosterone in human and pig skin in vitro. **Toxicology in Vitro**, *[S. l.]*, v. 48, p. 71–77, 2018 a. Disponível em: https://doi.org/10.1016/j.tiv.2017.12.014

HEYLINGS, Jon R.; DAVIES, Diane J.; BURTON, Rebecca. Dermal absorption of

testosterone in human and pig skin in vitro. **Toxicology in Vitro**, *[S. l.]*, v. 48, n. July 2017, p. 71–77, 2018 b. Disponível em: https://doi.org/10.1016/j.tiv.2017.12.014

HORITA, Daisuke; TODO, Hiroaki; SUGIBAYASHI, Kenji. Effect of Ethanol Pretreatment on Skin Permeation of Drugs. **Biological and Pharmaceutical Bulletin**, *[S. l.]*, v. 35, n. 8, p. 1343–1348, 2012. Disponível em: https://doi.org/10.1248/bpb.b12-00293

HOTLINE, HIV. **Hepatic effects of 17 alpha-alkylated anabolic-androgenic steroids**. *[S. l.: s. n.]*.

HOUGHTON, Ed; MAYNARD, Steve. Some aspects of doping and medication control in equine sports. **Doping in Sports: Biochemical Principles, Effects and Analysis**, *[S. l.]*, p. 369–409, 2010.

HOUK, Joy; GUY, Richard H. Membrane models for skin penetration studies. **Chemical Reviews**, *[S. l.]*, v. 88, n. 3, p. 455–472, 1988. Disponível em: https://doi.org/10.1021/cr00085a001

HUEBER, F.; SCHAEFER, H.; WEPIERRE, J. Role of transepidermal and transfollicular routes in percutaneous absorption of steroids: In vitro studies on human skin. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 7, n. 5, p. 237–244, 1994. Disponível em: https://doi.org/10.1159/000211300

HUEBER, Frédérique; WEPIERRE, Jacques; SCHAEFER, Hans. Role of transepidermal and transfollicular routes in percutaneous absorption of hydrocortisone and testosterone: in vivo study in the hairless rat. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 5, n. 2, p. 99–107, 1992.

HUTCHISON, J. Hormonal control of behaviour: steroid action in the brain. **Current Opinion in Neurobiology**, *[S. l.]*, v. 1, n. 4, p. 562–570, 1991. Disponível em: https://doi.org/10.1016/S0959-4388(05)80029-7

IACHINA, I. *et al.* The nanoscopic molecular pathway through human skin. **Biochimica et Biophysica Acta (BBA) - General Subjects**, *[S. l.]*, v. 1863, n. 7, p. 1226–1233, 2019. Disponível em: https://doi.org/10.1016/j.bbagen.2019.04.012

IM, Jueng Eun *et al.* Effect of application amounts on in vitro dermal absorption test using caffeine and testosterone. **Pharmaceutics**, *[S. l.]*, v. 13, n. 5, 2021. Disponível

em: https://doi.org/10.3390/pharmaceutics13050641

IMAI, Miko *et al.* Skin permeation of testosterone from viscoelastic lecithin reverse wormlike micellar solution. **Biological and Pharmaceutical Bulletin**, *[S. l.]*, v. 39, n. 4, p. 532–539, 2016. Disponível em: https://doi.org/10.1248/bpb.b15-00750

ISHII, Hiroshi; TODO, Hiroaki; SUGIBAYASHI, Kenji. Effect of Thermodynamic Activity on Skin Permeation and Skin Concentration of Triamcinolone Acetonide. **CHEMICAL & PHARMACEUTICAL BULLETIN**, *[S. l.]*, v. 58, n. 4, p. 556–561, 2010. Disponível em: https://doi.org/10.1248/cpb.58.556

IYER, R. *et al.* Pharmacokinetics of testosterone cream applied to scrotal skin. **Andrology**, *[S. l.]*, v. 5, n. 4, p. 725–731, 2017. Disponível em: https://doi.org/10.1111/andr.12357

JACOBI, Ute *et al.* Porcine ear skin: an in vitro model for human skin. **Skin Research and Technology**, *[S. l.]*, v. 13, n. 1, p. 19–24, 2007. Disponível em: https://doi.org/10.1111/j.1600-0846.2006.00179.x

JANŮŠOVÁ, Barbora *et al.* Effect of ceramide acyl chain length on skin permeability and thermotropic phase behavior of model stratum corneum lipid membranes. **Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids**, *[S. l.]*, v. 1811, n. 3, p. 129–137, 2011. Disponível em: https://doi.org/10.1016/j.bbalip.2010.12.003

JEEVAN, R.; VENKAT, R.; KHAN, Mansoor A. Effect of Menthol and Related Terpenes on the Percutaneous Absorption of Propranolol across Excised Hairless Mouse Skin. **Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 86, n. 12, p. 1369–1373, 1997.

JETZER, Walter E. *et al.* Permeation of Mouse Skin and Silicone Rubber Membranes by Phenols: Relationship to In Vitro Partitioning. **Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 75, n. 11, p. 1098–1103, 1986. Disponível em: https://doi.org/10.1002/jps.2600751116

JIANG, Qiudong *et al.* Development of essential oils as skin permeation enhancers: penetration enhancement effect and mechanism of action. **Pharmaceutical Biology**, *[S. l.]*, v. 55, n. 1, p. 1592–1600, 2017. Disponível em:

https://doi.org/10.1080/13880209.2017.1312464

JOCKENHÖVEL, F. Testosterone therapy - what, when and to whom? **The Aging Male**, *[S. l.]*, v. 7, n. 4, p. 319–324, 2004. Disponível em: https://doi.org/10.1080/13685530400016557

JORDAN, WP Jr. **Allergy and topical irritation associated with transdermal testosterone administration: a comparison of scrotal and nonscrotal transdermal systems**. *[S. l.: s. n.]*.

JUNG, Eui Chang; MAIBACH, Howard I. Animal models for percutaneous absorption. **Journal of Applied Toxicology**, *[S. l.]*, v. 35, n. 1, p. 1–10, 2015. Disponível em: https://doi.org/10.1002/jat.3004

KADIR, Ron; BARRY, Brian W. Alfa Bisabolol, a possible safe penetration enhancer for dermal and transdermal therapeutics. *[S. l.]*, v. 70, p. 87–94, 1991.

KAMATOU, Guy P. P. *et al.* Menthol: A simple monoterpene with remarkable biological properties. **Phytochemistry**, *[S. l.]*, v. 96, p. 15–25, 2013. Disponível em: https://doi.org/10.1016/j.phytochem.2013.08.005

KAMATOU, Guy P. P.; VILJOEN, Alvaro M. A Review of the Application and Pharmacological Properties of α-Bisabolol and α-Bisabolol-Rich Oils. **Journal of the American Oil Chemists' Society**, *[S. l.]*, v. 87, n. 1, p. 1–7, 2010. Disponível em: https://doi.org/10.1007/s11746-009-1483-3

KAMINETSKY, Jed; WYNIA, Blake. Current and emerging testosterone therapies for male hypogonadism. **Research and Reports in Endocrine Disorders**, *[S. l.]*, p. 59, 2015. Disponível em: https://doi.org/10.2147/RRED.S46193

KANG, Lifeng *et al.* Terpenes and Improvement of Transdermal Drug Delivery. *In*: **Natural Products**. Berlin, Heidelberg: Springer Berlin Heidelberg, 2013. p. 3757–3774. *E-book*. Disponível em: https://doi.org/10.1007/978-3-642-22144-6_160

KAO, Jack S. *et al.* Testosterone Perturbs Epidermal Permeability Barrier Homeostasis. **Journal of Investigative Dermatology**, *[S. l.]*, v. 116, n. 3, p. 443–451, 2001. Disponível em: https://doi.org/10.1046/j.1523-1747.2001.01281.x

KAPLUN-FRISCHOFF, Yael; TOUITOU, Elka. Testosterone skin permeation enhancement by menthol through formation of eutectic with drug and interaction with

skin lipids. **Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 86, n. 12, p. 1394–1399, 1997. Disponível em: https://doi.org/10.1021/js9701465

KARADZOVSKA, D.; BROOKS, J. D.; RIVIERE, J. E. Modeling the effect of experimental variables on the in vitro permeation of six model compounds across porcine skin. **International Journal of Pharmaceutics**, Center for Chemical Toxicology Research and Pharmacokinetics, North Carolina State University, Raleigh, NC 27607, United States, v. 443, n. 1–2, p. 58–67, 2013. Disponível em: https://doi.org/10.1016/j.ijpharm.2013.01.002

KARADZOVSKA, D.; RIVIERE, J. E. Assessing vehicle effects on skin absorption using artificial membrane assays. **European Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 50, n. 5, p. 569–576, 2013. Disponível em: https://doi.org/10.1016/j.ejps.2013.02.020

KAUFMAN, Joel M. Evolution of delivery systems for testosterone administration. **Current Sexual Health Reports**, *[S. l.]*, v. 1, n. 3, p. 109–114, 2004. Disponível em: https://doi.org/10.1007/s11930-004-0025-0

KAUFMANN, K.; DOHMEN, P. Adaption of a dermal in vitro method to investigate the uptake of chemicals across amphibian skin. **Environmental Sciences Europe**, *[S. l.]*, v. 28, n. 1, 2016. Disponível em: https://doi.org/10.1186/s12302-016-0080-y

KIM, MI-KYEONG; LEE, CHI-HO; KIM, DAE-DUK. Skin Permeation of Testosterone and its Ester Derivatives in Rats. **Journal of Pharmacy and Pharmacology**, *[S. l.]*, v. 52, n. 4, p. 369–375, 2000. Disponível em: https://doi.org/10.1211/0022357001774101

KIM, Mi Kyeong *et al.* Formulation of a reservoir-type testosterone transdermal delivery system. **International Journal of Pharmaceutics**, *[S. l.]*, v. 219, n. 1–2, p. 51–59, 2001. Disponível em: https://doi.org/10.1016/S0378-5173(01)00631-7

KIM, Saebom *et al.* Mechanisms of depigmentation by α-bisabolol. **Journal of Dermatological Science**, *[S. l.]*, v. 52, n. 3, p. 219–222, 2008. Disponível em: https://doi.org/10.1016/j.jdermsci.2008.06.005

KNORR, F. *et al.* Follicular transport route - Research progress and future perspectives. **European Journal of Pharmaceutics and Biopharmaceutics**, *[S. l.]*, v. 71, n. 2, p.

173–180, 2009 a. Disponível em: https://doi.org/10.1016/j.ejpb.2008.11.001

KNORR, Fanny *et al.* Follicular transport route–research progress and future perspectives. **European Journal of Pharmaceutics and Biopharmaceutics**, *[S. l.]*, v. 71, n. 2, p. 173–180, 2009 b.

KOCH, Christian; ULLAH, M. Iftekhar; RICHE, Daniel. Transdermal testosterone replacement therapy in men. **Drug Design, Development and Therapy**, *[S. l.]*, p. 101, 2014. Disponível em: https://doi.org/10.2147/DDDT.S43475

KOVÁČIK, Andrej; KOPEČNÁ, Monika; VÁVROVÁ, Kateřina. Permeation enhancers in transdermal drug delivery: benefits and limitations. **Expert Opinion on Drug Delivery**, *[S. l.]*, v. 17, n. 2, p. 145–155, 2020. Disponível em: https://doi.org/10.1080/17425247.2020.1713087

KRETSOS, K. *et al.* Partitioning, diffusivity and clearance of skin permeants in mammalian dermis. **International Journal of Pharmaceutics**, College of Pharmacy, University of Cincinnati Academic Health Center, P.O. Box 670004, Cincinnati, OH 45267-0004, United States, v. 346, n. 1–2, p. 64–79, 2008. Disponível em: https://doi.org/10.1016/j.ijpharm.2007.06.020

KRUGLUGER, Walter *et al.* Reorganization of hair follicles in human skin organ culture induced by cultured human follicle-derived cells. **Experimental dermatology**, *[S. l.]*, v. 14, n. 8, p. 580–585, 2005.

KÜCHLER, S.; STRÜVER, K.; FRIESS, W. Reconstructed skin models as emerging tools for drug absorption studies. **Expert Opinion on Drug Metabolism and Toxicology**, *[S. l.]*, v. 9, n. 10, p. 1255–1263, 2013. Disponível em: https://doi.org/10.1517/17425255.2013.816284

LADEMANN, J. *et al.* Which Skin Model Is the Most Appropriate for the Investigation of Topically Applied Substances into the Hair Follicles? **Skin Pharmacology and Physiology**, *[S. l.]*, v. 23, n. 1, p. 47–52, 2010. Disponível em: https://doi.org/10.1159/000257263

LAI, J.; MAIBACH, H. I. Experimental Models in Predicting Topical Antifungal Efficacy: Practical Aspects and Challenges. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 22, n. 5, p. 231–239, 2009. Disponível em: https://doi.org/10.1159/000235827

LAND, Laura M.; LI, Ping; BUMMER, Paul Michael. The Influence of Water Content of Triglyceride Oils on the Solubility of Steroids. **Pharmaceutical Research**, *[S. l.]*, v. 22, n. 5, p. 784–788, 2005. Disponível em: https://doi.org/10.1007/s11095-005-2595-6

LAOTHAWEERUNGSAWAT, Natnaree *et al.* Transdermal delivery enhancement of carvacrol from Origanum vulgare L. essential oil by microemulsion. **International Journal of Pharmaceutics**, *[S. l.]*, v. 579, p. 119052, 2020. Disponível em: https://doi.org/10.1016/j.ijpharm.2020.119052

LAUTERBACH, Andreas; MÜLLER-GOYMANN, Christel C. Applications and limitations of lipid nanoparticles in dermal and transdermal drug delivery via the follicular route. **European Journal of Pharmaceutics and Biopharmaceutics**, *[S. l.]*, v. 97, p. 152–163, 2015.

LEE, H. J.; CHANG, C. Recent advances in androgen receptor action. **Cellular and Molecular Life Sciences (CMLS)**, *[S. l.]*, v. 60, n. 8, p. 1613–1622, 2003. Disponível em: https://doi.org/10.1007/s00018-003-2309-3

LEICHTNAM, M. L. *et al.* Testosterone hormone replacement therapy: State-of-the-art and emerging technologies. **Pharmaceutical Research**, *[S. l.]*, v. 23, n. 6, p. 1117–1132, 2006 a. Disponível em: https://doi.org/10.1007/s11095-006-0072-5

LEICHTNAM, M. L. *et al.* Enhancement of transdermal testosterone delivery by supersaturation. **Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 95, n. 11, p. 2373–2379, 2006 b. Disponível em: https://doi.org/10.1002/jps.20669

LEICHTNAM, Marie Laure *et al.* Identification of penetration enhancers for testosterone transdermal delivery from spray formulations. **Journal of Controlled Release**, *[S. l.]*, v. 113, n. 1, p. 57–62, 2006 c. Disponível em: https://doi.org/10.1016/j.jconrel.2006.03.008

LEVANG, Angela K.; ZHAO, Kaidi; SINGH, Jagdish. Effect of ethanol/propylene glycol on the in vitro percutaneous absorption of aspirin, biophysical changes and macroscopic barrier properties of the skin. **International Journal of Pharmaceutics**, *[S. l.]*, v. 181, n. 2, p. 255–263, 1999. Disponível em: https://doi.org/10.1016/S0378-5173(99)00055-1

LI, Y.; KASHANGAKI, G. D.; YL, F. .. Interactions of testosterone and carbamazepine

with carvacrol mediated by CYP3A4. **Chinese Journal of Pharmacology and Toxicology**, *[S. l.]*, v. 34, p. 605–609, 2020.

LILLYWHITE, H. B. Water relations of tetrapod integument. **Journal of Experimental Biology**, *[S. l.]*, v. 209, n. 2, p. 202–226, 2006. Disponível em: https://doi.org/10.1242/jeb.02007

LIM, Perry Fung Chye; LIU, Xiang Yang; CHAN, Sui Yung. A Review on Terpenes as Skin Penetration Enhancers in Transdermal Drug Delivery. **Journal of Essential Oil Research**, *[S. l.]*, v. 21, n. 5, p. 423–428, 2009. Disponível em: https://doi.org/10.1080/10412905.2009.9700208

LIN, S. Y. *et al.* Comparisons of different animal skins with human skin in drug percutaneous penetration studies. **Methods and findings in experimental and clinical pharmacology**, *[S. l.]*, v. 14, n. 8, p. 645–654, 1992. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/1494304

LLEWELYN, Victoria K.; BERGER, Lee; GLASS, Beverley D. Permeability of frog skin to chemicals: effect of penetration enhancers. **Heliyon**, *[S. l.]*, v. 5, n. 8, p. e02127, 2019. Disponível em: https://doi.org/10.1016/j.heliyon.2019.e02127

LU, Wangding *et al.* Preparation and characterization of a metered dose transdermal spray for testosterone. **Acta Pharmaceutica Sinica B**, *[S. l.]*, v. 3, n. 6, p. 392–399, 2013. Disponível em: https://doi.org/10.1016/j.apsb.2013.10.003

MAGNUSSON, B. M. *et al.* Percutaneous Absorption of Steroids: Determination of in vitro Permeability and Tissue Reservoir Characteristics in Human Skin Layers. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 19, n. 6, p. 336–342, 2006. Disponível em: https://doi.org/10.1159/000095254

MAKIN, Hugh L. J. *et al.* General Methods for the Extraction, Purification, and Measurement of Steroids by Chromatography and Mass Spectrometry BT - Steroid Analysis. *In*: MAKIN, Hugh L. J.; GOWER, D. B. (org.). Dordrecht: Springer Netherlands, 2010. p. 163–282. *E-book*. Disponível em: https://doi.org/10.1023/b135931_3

MANGELSDORF, Susanne *et al.* Comparative study of hair follicle morphology in eight mammalian species and humans. **Skin Research and Technology**, *[S. l.]*, v. 20, n.

2, p. 147–154, 2014. Disponível em: https://doi.org/10.1111/srt.12098

MANNI, ANDREA *et al.* Bioavailability of Albumin-Bound Testosterone†. **The Journal of Clinical Endocrinology & Metabolism**, *[S. l.]*, v. 61, n. 4, p. 705–710, 1985. Disponível em: https://doi.org/10.1210/jcem-61-4-705

MARCHESE, Anna *et al.* Antibacterial and antifungal activities of thymol: A brief review of the literature. **Food Chemistry**, *[S. l.]*, v. 210, p. 402–414, 2016. Disponível em: https://doi.org/10.1016/j.foodchem.2016.04.111

MARKOVA, Marina S. *et al.* A Role for the Androgen Receptor in Collagen Content of the Skin. **Journal of Investigative Dermatology**, *[S. l.]*, v. 123, n. 6, p. 1052–1056, 2004. Disponível em: https://doi.org/10.1111/j.0022-202X.2004.23494.x

MARRAKCHI, Slaheddine; MAIBACH, Howard I. Biophysical parameters of skin: map of human face, regional, and age-related differences. **Contact Dermatitis**, *[S. l.]*, v. 57, n. 1, p. 28–34, 2007. Disponível em: https://doi.org/10.1111/j.1600-0536.2007.01138.x

MARTITS, A. M.; COSTA, E. M. .. Diretrizes em foco: Tratamento e Monitoramento da Andropausa. **Associação Médica Brasileira**, *[S. l.]*, v. 51, n. 5, p. 121–132, 2005.

MAZER, Norman A. New clinical applications of transdermal testosterone delivery in men and women. **Journal of Controlled Release**, *[S. l.]*, v. 65, n. 1–2, p. 303–315, 2000. Disponível em: https://doi.org/10.1016/S0168-3659(99)00252-7

MCFARLANE, R. M. *et al.* The design of a pedicle flap in the rat to study necrosis and its prevention. **Plastic and Reconstructive Surgery**, *[S. l.]*, v. 35, n. 2, p. 177–182, 1965. Disponível em: https://doi.org/10.1097/00006534-196502000-00007

MCNICHOLAS, T. A. *et al.* A novel testosterone gel formulation normalizes androgen levels in hypogonadal men, with improvements in body composition and sexual function. **BJU International**, *[S. l.]*, v. 91, n. 1, p. 69–74, 2003. Disponível em: https://doi.org/10.1046/j.1464-410X.2003.04016.x

MEIKLE, A. W. *et al.* Pharmacokinetics and metabolism of a permeation-enhanced testosterone transdermal system in hypogonadal men: Influence of application site - A clinical research center study. **Journal of Clinical Endocrinology and Metabolism**, Department of Medicine and Pathology, University of Utah, Salt Lake City, UT 84132,

United States, v. 81, n. 5, p. 1832–1840, 1996. Disponível em: https://doi.org/10.1210/jc.81.5.1832

MICHEL, Martine *et al.* Characterization of a new tissue-engineered human skin equivalent with hair. **In Vitro Cellular & Developmental Biology-Animal**, *[S. l.]*, v. 35, n. 6, p. 318, 1999.

MILLS, P. C. Vehicle effects on the In vitro penetration of testosterone through equine skin. **Veterinary Research Communications**, *[S. l.]*, v. 31, n. 2, p. 227–233, 2007. Disponível em: https://doi.org/10.1007/s11259-006-3446-6

MILLS, P. C.; MAGNUSSON, B. M.; CROSS, S. E. The effects of vehicle and region of application on in vitro penetration of testosterone through canine skin. **Veterinary Journal**, *[S. l.]*, v. 171, n. 2, p. 276–280, 2006. Disponível em: https://doi.org/10.1016/j.tvjl.2004.11.013

MISRA, A. *et al.* Formulation of a transdermal system for biphasic delivery of testosterone. **Journal of Controlled Release**, National Institute of Immunology, Aruna Asaf Ali Road, New Delhi 110067, India, v. 39, n. 1, p. 1–7, 1996. Disponível em: https://doi.org/10.1016/0168-3659(95)00122-0

MITRAGOTRI, Samir. Modeling skin permeability to hydrophilic and hydrophobic solutes based on four permeation pathways. **Journal of Controlled Release**, *[S. l.]*, v. 86, n. 1, p. 69–92, 2003. Disponível em: https://doi.org/10.1016/S0168-3659(02)00321-8

MOGHADAM, Shadi H. *et al.* Effect of Chemical Permeation Enhancers on Stratum Corneum Barrier Lipid Organizational Structure and Interferon Alpha Permeability. **Molecular Pharmaceutics**, *[S. l.]*, v. 10, n. 6, p. 2248–2260, 2013. Disponível em: https://doi.org/10.1021/mp300441c

MONTEIRO-RIVIERE, N. A. Comparative anatomy, physiology, and biochemistry of mammalian skin. *In*: HOBSON, D. .. (org.). **Dermal and Ocular Toxicology: Fundamentals and Methods**. Boca Raton: CRC Press, 1991. p. 3–72. *E-book.*

MOORADIAN, A. D.; MORLEY, J. E.; KORENMAN, S. G. Biological actions of androgens. **Endocrine Rev.**, *[S. l.]*, n. 8, p. 1–28, 1987.

MOSER, Katrin *et al.* Passive skin penetration enhancement and its quantification in

vitro. **European Journal of Pharmaceutics and Biopharmaceutics**, *[S. l.]*, v. 52, n. 2, p. 103–112, 2001. Disponível em: https://doi.org/10.1016/S0939-6411(01)00166-7

MÜNSTER, U. *et al.* Testosterone metabolism in human skin cells in vitro and its interaction with estradiol and dutasteride. **Skin Pharmacology and Applied Skin Physiology**, *[S. l.]*, v. 16, n. 6, p. 356–366, 2003. Disponível em: https://doi.org/10.1159/000072930

NAKAMURA, Motoki *et al.* Alternative test models for skin ageing research. **Experimental Dermatology**, *[S. l.]*, v. 27, n. 5, p. 495–500, 2018. Disponível em: https://doi.org/10.1111/exd.13519

NATIONAL RESOURCE CENTER. **Osteoporosis and Related Bone Diseases**. *[S. l.: s. n.]*. Disponível em: https://www.bones.nih.gov/health-info/bone/osteoporosis/men.

NETZLAFF, F. *et al.* Comparison of bovine udder skin with human and porcine skin in percutaneous permeation experiments. **ATLA Alternatives to Laboratory Animals**, *[S. l.]*, v. 34, n. 5, p. 499–513, 2006. Disponível em: https://www.scopus.com/inward/record.uri?eid=2-s2.0-33751503464&partnerID=40&md5=bbf62b84f6b5107f878afee64e6ff6a5

NEUPANE, Rabin *et al.* Alternatives to Biological Skin in Permeation Studies: Current Trends and Possibilities. **Pharmaceutics**, *[S. l.]*, v. 12, n. 2, p. 152, 2020. Disponível em: https://doi.org/10.3390/pharmaceutics12020152

NG, Shiow-Fern *et al.* Validation of a Static Franz Diffusion Cell System for In Vitro Permeation Studies. **AAPS PharmSciTech**, *[S. l.]*, v. 11, n. 3, p. 1432–1441, 2010. Disponível em: https://doi.org/10.1208/s12249-010-9522-9

NICOLAZZO, Joseph A. *et al.* Synergistic enhancement of testosterone transdermal delivery. **Journal of Controlled Release**, *[S. l.]*, v. 103, n. 3, p. 577–585, 2005. Disponível em: https://doi.org/10.1016/j.jconrel.2004.12.007

NIESCHLAG, E.; BEHRE, H. M. **Testosterone**. 2nd. ed. Berlin, Heidelberg: Springer Berlin Heidelberg, 1998. *E-book*. Disponível em: https://doi.org/10.1007/978-3-642-72185-4

NIESCHLAG, Eberhard. Current topics in testosterone replacement of hypogonadal men. **Best Practice & Research Clinical Endocrinology & Metabolism**, *[S. l.]*, v. 29,

n. 1, p. 77–90, 2015. Disponível em: https://doi.org/10.1016/j.beem.2014.09.008

NIU, Xiao-Qian *et al.* Mechanism investigation of ethosomes transdermal permeation. **International Journal of Pharmaceutics: X**, *[S. l.]*, v. 1, p. 100027, 2019. Disponível em: https://doi.org/10.1016/j.ijpx.2019.100027

NORMAN, Anthony W.; LITWACK, Gerald. **Hormones**. *[S. l.]*: Academic Press, 1997. *E-book*.

OECD. **Guideline for the testing of chemicals. Skin absorption: in vivo method**. *[S. l.: s. n.]*

OECD. **Draft guidance document for the conduct of skin absorption studies**. *[S. l.: s. n.]*

OECD. **Test Guidelines Program. Percutaneous absorption testing: is there a way to consensus?**. *[S. l.: s. n.]*

OECD. OECD guideline for the testing of chemicals, No. 431. *In*: OECD (org.). **In vitro skin corrosion: human skin model test.** Paris, France: OECD, 2004 a. p. 8. *E-book*.

OECD. OECD guideline for the testing of chemicals, No. 432. *In*: OECD (org.). **In vitro 3T3 NRU phototoxicity test**. Paris, France: OECD, 2004 b. p. 15. *E-book*.

OECD. **Guidance Document for the Conduct of Skin Absorption Studies**. *[S. l.]*: OECD, 2004 c. Disponível em: https://doi.org/10.1787/9789264078796-en

OGISO, Taro *et al.* Transfollicular Drug Delivery: Penetration of Drugs Through Human Scalp Skin and Comparison of Penetration Between Scalp and Abdominal Skins In Vitro. **Journal of Drug Targeting**, *[S. l.]*, v. 10, n. 5, p. 369–378, 2002. Disponível em: https://doi.org/10.1080/1061186021000001814

OKIMOTO, K.; RAJEWSKI, RA; STELLA, VJ. Release of testosterone from an osmotic pump tablet utilizing (SBE)7m-[beta]-cyclodextrin as both a solubilizing and an osmotic pump agent. **J Control Release**, *[S. l.]*, n. 58, p. 29–38, 1998.

ORIBA, H. A.; BUCKS, D. A. W.; MAIBACH, H. I. Percutaneous absorption of hydrocortisone and testosterone on the vulva and forearm: Effect of the menopause and site. **British Journal of Dermatology**, *[S. l.]*, v. 134, n. 2, p. 229–233, 1996.

Disponível em: https://doi.org/10.1111/j.1365-2133.1996.tb07606.x

ORWOLL, ERIC S.; KLEIN, ROBERT F. Osteoporosis in Men. **Endocrine Reviews**, *[S. l.]*, v. 16, n. 1, p. 87–116, 1995. Disponível em: https://doi.org/10.1210/edrv-16-1-87

OTBERG, Nina *et al.* The role of hair follicles in the percutaneous absorption of caffeine. **British Journal of Clinical Pharmacology**, *[S. l.]*, v. 65, n. 4, p. 488–492, 2008. Disponível em: https://doi.org/10.1111/j.1365-2125.2007.03065.x

OTTAVIANI, G.; MARTEL, S.; CARRUPT, P. A. Parallel artificial membrane permeability assay: A new membrane for the fast prediction of passive human skin permeability. **Journal of Medicinal Chemistry**, *[S. l.]*, v. 49, n. 13, p. 3948–3954, 2006. Disponível em: https://doi.org/10.1021/jm060230+

PABLA, Dimple; ZIA, Hossein. A comparative permeation/release study of different testosterone gel formulations. **Drug Delivery**, *[S. l.]*, v. 14, n. 6, p. 389–396, 2007. Disponível em: https://doi.org/10.1080/10717540701203075

PALMER, Brian; DELOUISE, Lisa. Nanoparticle-Enabled Transdermal Drug Delivery Systems for Enhanced Dose Control and Tissue Targeting. **Molecules**, *[S. l.]*, v. 21, n. 12, p. 1719, 2016. Disponível em: https://doi.org/10.3390/molecules21121719

PANCHAGNULA, Ramesh *et al.* Transdermal delivery of naloxone: Effect of water, propylene glycol, ethanol and their binary combinations on permeation through rat skin. **International Journal of Pharmaceutics**, *[S. l.]*, v. 219, n. 1–2, p. 95–105, 2001. Disponível em: https://doi.org/10.1016/S0378-5173(01)00634-2

PARDRIDGE, William M. Serum bioavailability of sex steroid hormones. **Clinics in Endocrinology and Metabolism**, *[S. l.]*, v. 15, n. 2, p. 259–278, 1986. Disponível em: https://doi.org/10.1016/S0300-595X(86)80024-X

PARK, Juyoung; LEE, Handule; PARK, Kwangsik. Mixture Toxicity of Methylisothiazolinone and Propylene Glycol at a Maximum Concentration for Personal Care Products. **Toxicological Research**, *[S. l.]*, v. 34, n. 4, p. 355–361, 2018. Disponível em: https://doi.org/10.5487/TR.2018.34.4.355

PATEL, Tejesh; ISHIUJI, Yozo; YOSIPOVITCH, Gil. Menthol: A refreshing look at this ancient compound. **Journal of the American Academy of Dermatology**, *[S. l.]*, v. 57, n. 5, p. 873–878, 2007. Disponível em: https://doi.org/10.1016/j.jaad.2007.04.008

PATZELT, Alexa; LADEMANN, Juergen. Drug delivery to hair follicles. **Expert Opinion on Drug Delivery**, *[S. l.]*, v. 10, n. 6, p. 787–797, 2013. Disponível em: https://doi.org/10.1517/17425247.2013.776038

PELLETIER, Georges; REN, L. Localization of sex steroid receptors in human skin. **Histology and Histopathology**, *[S. l.]*, v. 19, n. 2, p. 629–636, 2004. Disponível em: https://doi.org/10.14670/HH-19.629

PERBELLINI, Luigi *et al.* Determination of alpha-bisabolol in human blood by micro-HPLC–ion trap MS and head space-GC–MS methods. **Journal of Chromatography B**, *[S. l.]*, v. 812, n. 1–2, p. 373–377, 2004. Disponível em: https://doi.org/10.1016/j.jchromb.2004.07.046

PLANZ, Viktoria; LEHR, Claus Michael; WINDBERGS, Maike. In vitro models for evaluating safety and efficacy of novel technologies for skin drug delivery. **Journal of Controlled Release**, *[S. l.]*, v. 242, p. 89–104, 2016. Disponível em: https://doi.org/10.1016/j.jconrel.2016.09.002

PLUMB, D. C. **Meclizine. Veterinary Drug Book, ed 4. Ames**. *[S. l.]*: Iowa State University Press, 2002.

POLSON, Cara *et al.* Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry. **Journal of Chromatography B**, *[S. l.]*, v. 785, n. 2, p. 263–275, 2003. Disponível em: https://doi.org/10.1016/S1570-0232(02)00914-5

PONEC, M. *et al.* Barrier Function in Reconstructed Epidermis and Its Resemblance to Native Human Skin. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 14, n. 1, p. 63–71, 2001. Disponível em: https://doi.org/10.1159/000056392

PONEC, Maria *et al.* Lipid and ultrastructural characterization of reconstructed skin models. **International Journal of Pharmaceutics**, *[S. l.]*, v. 203, n. 1–2, p. 211–225, 2000. Disponível em: https://doi.org/10.1016/S0378-5173(00)00459-2

PONEC, Maria *et al.* Characterization of Reconstructed Skin Models. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 15, n. 1, p. 4–17, 2002. Disponível em: https://doi.org/10.1159/000066682

POULIOT, Roxane *et al.* Physical characterization of the stratum corneum of an in vitro

human skin equivalent produced by tissue engineering and its comparison with normal human skin by ATR-FTIR spectroscopy and thermal analysis (DSC). **Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids**, *[S. l.]*, v. 1439, n. 3, p. 341–352, 1999. Disponível em: https://doi.org/10.1016/S1388-1981(99)00086-4

POURFARZAD, Amir; AHMADIAN, Zahra; HABIBI-NAJAFI, Mohammad B. Interactions between polyols and wheat biopolymers in a bread model system fortified with inulin: A Fourier transform infrared study. **Heliyon**, *[S. l.]*, v. 4, n. 12, p. e01017, 2018. Disponível em: https://doi.org/10.1016/j.heliyon.2018.e01017

PRAUSNITZ, M. R.; LANGER, R. Transdermal drug delivery. **Nature Biotechnology**, *[S. l.]*, v. 26, n. 11, p. 1261–1268, 2008. Disponível em: https://doi.org/10.1038/nbt.1504

QVIST, M. H. *et al.* Evaluation of Gottingen minipig skin for transdermal in vitro permeation studies. **European Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 11, n. 1, p. 59–68, 2000. Disponível em: https://doi.org/10.1016/S0928-0987(00)00091-9

RANEY, Sam G. *et al.* Pharmacokinetics-Based Approaches for Bioequivalence Evaluation of Topical Dermatological Drug Products. **Clinical Pharmacokinetics**, *[S. l.]*, v. 54, n. 11, p. 1095–1106, 2015. Disponível em: https://doi.org/10.1007/s40262-015-0292-0

RANG, H. P. *et al.* O sistema reprodutor. *In*: **Rang & Dale: Farmacologia.** 8. ed. ed. Rio de Janeiro: Elsevie, 2016. *E-book*.

RIGGS, B. Lawrence; KHOSLA, Sundeep; MELTON, L. Joseph. Sex Steroids and the Construction and Conservation of the Adult Skeleton. **Endocrine Reviews**, *[S. l.]*, v. 23, n. 3, p. 279–302, 2002. Disponível em: https://doi.org/10.1210/edrv.23.3.0465

ROBERTS, Michael S. *et al.* Topical drug delivery: History, percutaneous absorption, and product development. **Advanced Drug Delivery Reviews**, *[S. l.]*, v. 177, p. 113929, 2021. Disponível em: https://doi.org/10.1016/j.addr.2021.113929

RODGER, Alison; SANDERS, Karen. UV-Visible Absorption Spectroscopy, Biomacromolecular Applications. *In*: **Encyclopedia of Spectroscopy and Spectrometry**. *[S. l.]*: Elsevier, 2017. p. 495–502. *E-book*. Disponível em: https://doi.org/10.1016/B978-0-12-803224-4.00108-4

ROUGIER, André; LOTTE, Claire; MAIBACH, Howard I. In Vivo Percutaneous Penetration of Some Organic Compounds Related to Anatomic Site in Humans: Predictive Assessment by the Stripping Method. **Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 76, n. 6, p. 451–454, 1987. Disponível em: https://doi.org/10.1002/jps.2600760608

SANDBERG, Avery A.; SLAUNWHITE, W. Roy. METABOLISM OF 4-C14-TESTOSTERONE IN HUMAN SUBJECTS. I. DISTRIBUTION IN BILE, BLOOD, FECES AND URINE. **Journal of Clinical Investigation**, *[S. l.]*, v. 35, n. 12, p. 1331–1339, 1956. Disponível em: https://doi.org/10.1172/JCI103389

SANDBY-MØLLER, Jane; POULSEN, Thomas; WULF, Hans Christian. Epidermal Thickness at Different Body Sites: Relationship to Age, Gender, Pigmentation, Blood Content, Skin Type and Smoking Habits. **Acta Dermato-Venereologica**, *[S. l.]*, v. 83, n. 6, p. 410–413, 2003. Disponível em: https://doi.org/10.1080/00015550310015419

SAPRA, Bharti; JAIN, Subheet; TIWARY, A. K. Percutaneous permeation enhancement by terpenes: Mechanistic view. **AAPS Journal**, *[S. l.]*, v. 10, n. 1, p. 120–132, 2008. Disponível em: https://doi.org/10.1208/s12248-008-9012-0

SATO, Kiyoshi; SUGIBAYASHI, Kenji; MORIMOTO, Yasunori. Species Differences in Percutaneous Absorption of Nicorandil. **Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 80, n. 2, p. 104–107, 1991. Disponível em: https://doi.org/10.1002/jps.2600800203

SCCS. Basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients. **Eur. Comm**, *[S. l.]*, p. 1–14, 2010.

SCHÄFER-KORTING, M. *et al.* Reconstructed human epidermis for skin absorption testing: Results of the German prevalidation study. **ATLA Alternatives to Laboratory Animals**, *[S. l.]*, v. 34, n. 3, p. 283–294, 2006. Disponível em: https://www.scopus.com/inward/record.uri?eid=2-s2.0-33746311297&partnerID=40&md5=34f8b2ff2a4c08d6e4709c3969b113b9

SCHÄFER-KORTING, Monika *et al.* The Use of Reconstructed Human Epidermis for Skin Absorption Testing: Results of the Validation Study. **Alternatives to Laboratory Animals**, *[S. l.]*, v. 36, n. 2, p. 161–187, 2008. Disponível em: https://doi.org/10.1177/026119290803600207

SCHLUPP, P. *et al.* Development and validation of an alternative disturbed skin model by mechanical abrasion to study drug penetration. **Results in Pharma Sciences**, *[S. l.]*, v. 4, p. 26–33, 2014. Disponível em: https://doi.org/10.1016/j.rinphs.2014.09.002

SCHMOOK, Fritz P.; MEINGASSNER, Josef G.; BILLICH, Andreas. Comparison of human skin or epidermis models with human and animal skin in in-vitro percutaneous absorption. **International Journal of Pharmaceutics**, *[S. l.]*, v. 215, n. 1–2, p. 51–56, 2001. Disponível em: https://doi.org/10.1016/S0378-5173(00)00665-7

SCHREIBER, S. *et al.* Reconstructed epidermis versus human and animal skin in skin absorption studies. **Toxicology in Vitro**, *[S. l.]*, v. 19, n. 6, p. 813–822, 2005. Disponível em: https://doi.org/10.1016/j.tiv.2005.04.004

SCHWARB, Fabian P. *et al.* Effect of Concentration and Degree of Saturation of Topical Fluocinonide Formulations on In Vitro Membrane Transport and In Vivo Availability on Human Skin. **Pharmaceutical Research**, *[S. l.]*, v. 16, n. 6, p. 909–915, 1999. Disponível em: https://doi.org/10.1023/A:1018890422825

SCHWARZ, Dennis H.; ENGELKE, Annegret; WENZ, Gerhard. Solubilizing steroidal drugs by β-cyclodextrin derivatives. **International Journal of Pharmaceutics**, *[S. l.]*, v. 531, n. 2, p. 559–567, 2017. Disponível em: https://doi.org/10.1016/j.ijpharm.2017.07.046

SHAH, D. K.; KHANDAVILLI, S.; PANCHAGNULA, R. Alteration of skin hydration and its barrier function by vehicle and permeation enhancers: A study using TGA, FTIR, TEWL and drug permeation as markers. **Methods and Findings in Experimental and Clinical Pharmacology**, *[S. l.]*, v. 30, n. 7, p. 499, 2008. Disponível em: https://doi.org/10.1358/mf.2008.30.7.1159653

SHAH, Vinod P.; MAIBACH, Howard I.; JENNER, John. **Topical Drug Bioavailability, Bioequivalence, and Penetration**. New York, NY: Springer New York, 2014. *E-book*. Disponível em: https://doi.org/10.1007/978-1-4939-1289-6

SHAHANI, S. *et al.* Androgens and erythropoiesis: Past and present. **Journal of Endocrinological Investigation**, *[S. l.]*, v. 32, n. 8, p. 704–716, 2009. Disponível em: https://doi.org/10.1007/BF03345745

SHAHIDI, N. A review of the chemistry, biological action, and clinical applications of

anabolic-androgenic steroids. **Clinical Therapeutics**, *[S. l.]*, v. 23, n. 9, p. 1355–1390, 2001. Disponível em: https://doi.org/10.1016/S0149-2918(01)80114-4

SHAPIRO, Jerry; PRICE, Vera H. Hair regrowth. Therapeutic agents. **Dermatologic Clinics**, *[S. l.]*, v. 16, n. 2, p. 341–356, 1998. Disponível em: https://doi.org/10.1016/S0733-8635(05)70017-6

SHARIFI-RAD, Mehdi *et al.* Carvacrol and human health: A comprehensive review. **Phytotherapy Research**, *[S. l.]*, v. 32, n. 9, p. 1675–1687, 2018. Disponível em: https://doi.org/10.1002/ptr.6103

SHIN, Jung-Won *et al.* Molecular Mechanisms of Dermal Aging and Antiaging Approaches. **International Journal of Molecular Sciences**, *[S. l.]*, v. 20, n. 9, p. 2126, 2019. Disponível em: https://doi.org/10.3390/ijms20092126

SHOSKES, J. J.; WILSON, M. K.; SPINNER, M. L. Pharmacology of testosterone replacement therapy preparations. **Translational Andrology and Urology**, *[S. l.]*, v. 5, n. 6, p. 834–843, 2016. Disponível em: https://doi.org/10.21037/tau.2016.07.10

SILVA, C. L. *et al.* Thermal Behaviour of Human Stratum Corneum. A differential scanning calorimetry study at high scanning rates. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 19, n. 3, p. 132–139, 2006 a. Disponível em: https://doi.org/10.1159/000092593

SILVA, C. L. *et al.* Study of human stratum corneum and extracted lipids by thermomicroscopy and DSC. **Chemistry and Physics of Lipids**, *[S. l.]*, v. 140, n. 1–2, p. 36–47, 2006 b. Disponível em: https://doi.org/10.1016/j.chemphyslip.2006.01.004

SILVA, Janine Passos Lima *et al.* Óleo essencial de orégano: interferência da composição química na atividade frente a Salmonella Enteritidis. **Ciência e Tecnologia de Alimentos**, *[S. l.]*, v. 30, p. 136–141, 2010. Disponível em: https://doi.org/10.1590/S0101-20612010000500021

SIMARD, M. *et al.* Modulation of the Lipid Profile of Reconstructed Skin Substitutes after Essential Fatty Acid Supplementation Affects Testosterone Permeability. **Cells**, *[S. l.]*, v. 8, n. 10, 2019. Disponível em: https://doi.org/10.3390/cells8101142

SIMON, Gad A.; MAIBACH, Howard I. The Pig as an Experimental Animal Model of Percutaneous Permeation in Man: Qualitative and Quantitative Observations – An

Overview. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 13, n. 5, p. 229–234, 2000. Disponível em: https://doi.org/10.1159/000029928

SINKÓ, Bálint *et al.* Skin-PAMPA: A new method for fast prediction of skin penetration. **European Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 45, n. 5, p. 698–707, 2012. Disponível em: https://doi.org/10.1016/j.ejps.2012.01.011

SJÖVALL, Peter *et al.* Imaging the distribution of skin lipids and topically applied compounds in human skin using mass spectrometry. **Scientific Reports**, *[S. l.]*, v. 8, n. 1, p. 16683, 2018. Disponível em: https://doi.org/10.1038/s41598-018-34286-x

SLIVKA, Sandra R. Testosterone metabolism in an in vitro skin model. **Cell Biology and Toxicology**, *[S. l.]*, v. 8, n. 4, p. 267–276, 1992. Disponível em: https://doi.org/10.1007/BF00156735

SMITH, JG; FISHER, RW; BLANK, H. The epidermal barrier: a comparison between scrotal and abdominal skin. **J Invest Dermatol**, *[S. l.]*, n. 36, p. 337–343, 1961.

SNOW, David H. Anabolic Steroids. **Veterinary Clinics of North America: Equine Practice**, *[S. l.]*, v. 9, n. 3, p. 563–576, 1993. Disponível em: https://doi.org/10.1016/S0749-0739(17)30386-3

SNYDER, Peter J. Chapter 45: Androgens and the Male Reproductive Tract. *In*: **Goodman and Gilman's The Pharmacological Basis of Therapeutics**. 13. ed. *[S. l.]*: McGraw-Hill Education, 2018. *E-book*.

SONGKRO, S.; BECKET, G.; RADES, T. The effects of p-menthane monoterpenes and related compounds on the percutaneous absorption of propranolol hydrochloride across newborn pig skin: III. Correlation between penetration enhancing activity and physicochemical properties of terpenes determined. **Journal of Drug Delivery Science and Technology**, *[S. l.]*, v. 14, n. 3, p. 223–228, 2004. Disponível em: https://doi.org/10.1016/S1773-2247(04)50104-1

SONGKRO, S.; RADES, T.; BECKET, G. Effects of some terpenes on the in vitro permeation of LHRH through newborn pig skin. **Die Pharmazie**, *[S. l.]*, v. 64, n. 2, p. 110–115, 2009 a. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/19320284

SONGKRO, S.; RADES, T.; BECKET, Gordon. Effects of some terpenes on the in vitro permeation of LHRH through newborn pig skin. **Die Pharmazie-An**

International Journal of Pharmaceutical Sciences, *[S. l.]*, v. 64, n. 2, p. 110–115, 2009 b.

SRINIVASAN, Balaji *et al.* TEER Measurement Techniques for In Vitro Barrier Model Systems. **Journal of Laboratory Automation**, *[S. l.]*, v. 20, n. 2, p. 107–126, 2015. Disponível em: https://doi.org/10.1177/2211068214561025

STAHL, J. *et al.* The in vitro use of the hair follicle closure technique to study the follicular and percutaneous permeation of topically applied drugs. **ATLA Alternatives to Laboratory Animals**, *[S. l.]*, v. 40, n. 1, p. 51–57, 2012. Disponível em: https://www.scopus.com/inward/record.uri?eid=2-s2.0-84860895398&partnerID=40&md5=d3fa071886903bd0d7be19bb9e6e6565

STENKEN, Julie A. *et al.* How Minimally Invasive is Microdialysis Sampling? A Cautionary Note for Cytokine Collection in Human Skin and other Clinical Studies. **The AAPS Journal**, *[S. l.]*, v. 12, n. 1, p. 73–78, 2010. Disponível em: https://doi.org/10.1208/s12248-009-9163-7

SUPE, Shibani; TAKUDAGE, Pooja. Methods for evaluating penetration of drug into the skin: A review. **Skin Research and Technology**, *[S. l.]*, v. 27, n. 3, p. 299–308, 2021. Disponível em: https://doi.org/10.1111/srt.12968

SURBER, Christian; SCHWARB, Fabian P.; SMITH, Eric W. Tape-stripping technique. **Journal of Toxicology: Cutaneous and Ocular Toxicology**, *[S. l.]*, v. 20, n. 4, p. 461–474, 2001. Disponível em: https://doi.org/10.1081/CUS-120001870

SWARBRICK, James; LEE, Geoffrey; BROM, Jeffrey. Drug Permeation through Human Skin: I. Effects of Storage Conditions of Skin. **Journal of Investigative Dermatology**, *[S. l.]*, v. 78, n. 1, p. 63–66, 1982. Disponível em: https://doi.org/10.1111/1523-1747.ep12497937

SWERDLOFF, Ronald S. *et al.* Long-Term Pharmacokinetics of Transdermal Testosterone Gel in Hypogonadal Men 1. **The Journal of Clinical Endocrinology & Metabolism**, *[S. l.]*, v. 85, n. 12, p. 4500–4510, 2000. Disponível em: https://doi.org/10.1210/jcem.85.12.7045

THOMAS, B. J.; FINNIN, B. C. The transdermal revolution. **Drug Discovery Today**, *[S. l.]*, v. 9, n. 16, p. 697–703, 2004. Disponível em: https://doi.org/10.1016/S1359-

6446(04)03180-0

THOMAS, Narisetty Sunil; PANCHAGNULA, Ramesh. Transdermal delivery of zidovudine: effect of vehicles on permeation across rat skin and their mechanism of action. **European Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 18, n. 1, p. 71–79, 2003. Disponível em: https://doi.org/10.1016/S0928-0987(02)00242-7

TODO, Hiroaki; MOHD, Fadli. Related Topic: Drug Permeation Through Hair Follicles. *In*: **Skin Permeation and Disposition of Therapeutic and Cosmeceutical Compounds**. Tokyo: Springer Japan, 2017. p. 399–409. *E-book*. Disponível em: https://doi.org/10.1007/978-4-431-56526-0_36

U.S. FOOD AND DRUG ADMINISTRATION. **Citizen petition denial response from FDA CDER to public citizen**. *[s. l.]*, 2014. Disponível em: www.regulations.%0Agov/document?D=FDA-2014-P-0258-0003. Acesso em: 26 fev. 2021.

VAN DE SANDT, J. J. M. *et al.* In vitro predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study. **Regulatory Toxicology and Pharmacology**, *[S. l.]*, v. 39, n. 3, p. 271–281, 2004. Disponível em: https://doi.org/10.1016/j.yrtph.2004.02.004

VAN GELE, M. *et al.* Three-dimensional skin models as tools for transdermal drug delivery: Challenges and limitations. **Expert Opinion on Drug Delivery**, Ghent University Hospital, Department of Dermatology, De Pintelaan 185, P6, B-9000 Ghent, Belgium, v. 8, n. 6, p. 705–720, 2011. Disponível em: https://doi.org/10.1517/17425247.2011.568937

VAN RAVENZWAAY, B.; LEIBOLD, E. A comparison between in vitro rat and human and in vivo rat skin absorption studies. **Human & Experimental Toxicology**, *[S. l.]*, v. 23, n. 9, p. 421–430, 2004. Disponível em: https://doi.org/10.1191/0960327104ht471oa

VASHISTH, Indu *et al.* Investigating the potential of essential oils as penetration enhancer for transdermal losartan delivery: Effectiveness and mechanism of action. **Asian Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 9, n. 5, p. 260–267, 2014. Disponível em: https://doi.org/10.1016/j.ajps.2014.06.007

VERMA, Amit *et al.* Transfollicular drug delivery: current perspectives. **Research and Reports in Transdermal Drug Delivery**, *[S. l.]*, p. 1, 2016. Disponível em: https://doi.org/10.2147/RRTD.S75809

VERYSER, L. *et al.* The influence of the Acyl chain on the transdermal penetration-enhancing effect of synthetic phytoceramides. **Skin Pharmacology and Physiology**, *[S. l.]*, v. 28, n. 3, p. 124–136, 2015. Disponível em: https://doi.org/10.1159/000365730

VILLEGAS, León F. *et al.* (+)- ep i -α-Bisbolol Is the Wound-Healing Principle of Peperomia ga lioides : Investigation of the in Vivo Wound-Healing Activity of Related Terpenoids. **Journal of Natural Products**, *[S. l.]*, v. 64, n. 10, p. 1357–1359, 2001. Disponível em: https://doi.org/10.1021/np0102859

WALTERS, K. A.; ROBERTS, M. .. The structure and function of skin. *In*: DEKKER, Marcel (org.). **Dermatological and Transdermal Formulations**. New York: *[s. n.]*, 2002. p. 1–40. *E-book*.

WANG, C. *et al.* Pharmacokinetics of Transdermal Testosterone Gel in Hypogonadal Men: Application of Gel at One Site Versus Four Sites: A General Clinical Research Center Study*. **The Journal of Clinical Endocrinology & Metabolism**, *[S. l.]*, v. 85, n. 3, p. 964–969, 2000. Disponível em: https://doi.org/10.1210/jcem.85.3.6437

WEI, Jonathan C. J. *et al.* Allometric scaling of skin thickness, elasticity, viscoelasticity to mass for micro-medical device translation: from mice, rats, rabbits, pigs to humans. **Scientific Reports**, *[S. l.]*, v. 7, n. 1, p. 15885, 2017. Disponível em: https://doi.org/10.1038/s41598-017-15830-7

WHO. **Dermal absorption**. *[S. l.]*: World Health Organization, 2006. *E-book*.

WILKINSON, S. C. *et al.* Interactions of skin thickness and physicochemical properties of test compounds in percutaneous penetration studies. **International Archives of Occupational and Environmental Health**, *[S. l.]*, v. 79, n. 5, p. 405–413, 2006 a. Disponível em: https://doi.org/10.1007/s00420-005-0056-5

WILKINSON, Simon C. *et al.* Interactions of skin thickness and physicochemical properties of test compounds in percutaneous penetration studies. **International Archives of Occupational and Environmental Health**, *[S. l.]*, v. 79, n. 5, p. 405–413, 2006 b. Disponível em: https://doi.org/10.1007/s00420-005-0056-5

WILLIAMS, A. C.; BARRY, B. W. Terpenes and the lipid-protein-partitioning theory of skin penetration enhancement. **Pharmaceutical Research**, *[S. l.]*, v. 8, n. 1, p. 17–24, 1991.

WILLIAMS, A. C.; BARRY, B. W. Skin absorption enhancers. **Crit. Rev. Ther. Drug Carrier Systems**, *[S. l.]*, n. 9, p. 305–353, 1992.

WILLIAMS, Adrian C.; BARRY, Brian W. Penetration enhancers. **Advanced Drug Delivery Reviews**, *[S. l.]*, v. 64, p. 128–137, 2012. Disponível em: https://doi.org/10.1016/j.addr.2012.09.032

WONG, Nicholas; LEVY, Miles; STEPHENSON, Iain. Hypogonadism in the HIV-Infected Man. **Current Treatment Options in Infectious Diseases**, *[S. l.]*, v. 9, n. 1, p. 104–116, 2017. Disponível em: https://doi.org/10.1007/s40506-017-0110-3

XING, Qing-Feng; LIN, Senshang; CHIEN, Yie W. Transdermal testosterone delivery in castrated Yucatan minipigs: pharmacokinetics and metabolism. **Journal of controlled release**, *[S. l.]*, v. 52, n. 1–2, p. 89–98, 1998.

YADAV, Ganapati D.; KAMBLE, Shashikant B. Synthesis of carvacrol by Friedel-Crafts alkylation of o-cresol with isopropanol using superacidic catalyst UDCaT-5. **Journal of Chemical Technology & Biotechnology**, *[S. l.]*, v. 84, n. 10, p. 1499–1508, 2009. Disponível em: https://doi.org/10.1002/jctb.2210

YAZAKI, Kazufumi; ARIMURA, Gen-ichiro; OHNISHI, Toshiyuki. 'Hidden' Terpenoids in Plants: Their Biosynthesis, Localization and Ecological Roles. **Plant and Cell Physiology**, *[S. l.]*, v. 58, n. 10, p. 1615–1621, 2017. Disponível em: https://doi.org/10.1093/pcp/pcx123

YU, Yi-Qun *et al.* Enhancing Permeation of Drug Molecules Across the Skin via Delivery in Nanocarriers: Novel Strategies for Effective Transdermal Applications. **Frontiers in Bioengineering and Biotechnology**, *[S. l.]*, v. 9, 2021. Disponível em: https://doi.org/10.3389/fbioe.2021.646554

YUN, Ye Eun *et al.* Artificial skin models for animal-free testing. **Journal of Pharmaceutical Investigation**, *[S. l.]*, v. 48, n. 2, p. 215–223, 2018. Disponível em: https://doi.org/10.1007/s40005-018-0389-1

ZHANG, D. *et al.* Evaluations of imidazolium ionic liquids as novel skin permeation

enhancers for drug transdermal delivery. **Pharmaceutical Development and Technology**, School of Life Science and Technology, Kunming University of Science and Technology, Kunming, China, v. 22, n. 4, p. 511–520, 2017. Disponível em: https://doi.org/10.3109/10837450.2015.1131718

ZHANG, Li-Wen; FANG, Yi-Ping; FANG, Jia-You. Enhancement techniques for improving 5-aminolevulinic acid delivery through the skin. **Dermatologica Sinica**, *[S. l.]*, v. 29, n. 1, p. 1–7, 2011. Disponível em: https://doi.org/10.1016/j.dsi.2011.02.002

ZHOU, Liping *et al.* Development of a high throughput equilibrium solubility assay using miniaturized shake-flask method in early drug discovery. **Journal of Pharmaceutical Sciences**, *[S. l.]*, v. 96, n. 11, p. 3052–3071, 2007. Disponível em: https://doi.org/10.1002/jps.20913

ZIMOCH, Jakub *et al.* Bio-engineering a prevascularized human tri-layered skin substitute containing a hypodermis. **Acta Biomaterialia**, *[S. l.]*, v. 134, p. 215–227, 2021. Disponível em: https://doi.org/10.1016/j.actbio.2021.07.033

ZSIKÓ, Stella *et al.* Methods to evaluate skin penetration in vitro. **Scientia Pharmaceutica**, *[S. l.]*, v. 87, n. 3, p. 19, 2019.

3. CAPÍTULO II – COMO PLANEJAR ENSAIOS DE PERMEAÇÃO CUTÂNEA PARA FÁRMACOS QUE APRESENTAM LIMITAÇÕES BIOFARMACÊUTICAS COMO A TESTOSTERONA?

Neste capítulo, estudaram-se como condições experimentais tais como espessura de pele, modelo animal, composição das fases doadora e receptora, concentração do fármaco na formulação, índice de supersaturação, tempo do ensaio, quantificação das amostras e retenção cutânea do fármaco afetam a permeação cutânea da testosterona em ensaios realizados em células de difusão do tipo Franz. Este fármaco foi selecionado por apresentar limitações biofarmacêuticas (baixa solubilidade e permeação percutânea) e devido a sua ampla utilização em formulações transdérmicas. O produto deste capítulo é um artigo científico de revisão, que foi publicado na revista *International Journal of Pharmaceutics*.

3.1. ARTIGO CIENTÍFICO I

HOW TO DEFINE THE EXPERIMENTAL CONDITIONS OF SKIN PERMEATION ASSAYS FOR DRUGS PRESENTING BIOPHARMACEUTICAL LIMITATIONS? THE EXPERIENCE WITH TESTOSTERONE

Nicole Esposto Biondo; Débora Fretes Argenta; Gabriela Schneider Rauber; Thiago Caon*

Postgraduate Program in Pharmacy (PGFAR), Federal University of Santa Catarina, Trindade, 88040-900 Florianopolis, SC, Brazil.

*Corresponding author: T. Caon

Laboratory of Pharmaceutical Technology & Cosmetology
Department of Pharmaceutical Sciences
Federal University of Santa Catarina (www.ufsc.br)
Telephone number: +55 48 3721 5850
E-mail: caon.thiago@ufsc.br / thiagocaon@gmail.com
ORCID: 0000-0003-3030-6310

ABSTRACT

Cutaneous permeation assays are crucial to attest the performance or bioequivalence of topical or transdermal products. Although the official guidelines (e.g., FDA/EMA) play a key role in harmonizing the experimental design, alternative methods are often proposed by the scientific community, which makes it difficult to compare results from different studies. In this review, permeation assays with testosterone (TST) were selected to show this high variability in drug transport rate. The main sources of variation discussed were tissue thickness, animal model, donor and receptor fluid constitution, type of solubilizing agent used in aqueous fluids, drug concentration, degree of supersaturation, skin lipid content, number of experimental times and the physical-chemical stability of the molecule in test fluids. This variation becomes even more critical for molecules that present biopharmaceutical limitations such as TST. In addition, the skin presents specific receptors for this hormone due to its physiological action in this region of the body, which makes the evaluation of the TST transport rate in this tissue even more challenging. The impact of each experimental parameter mentioned above on the flux or permeation coefficient of TST is discussed in detail in the review. Assays used to evaluate tissue integrity are also presented.

Keywords: permeation studies; Franz-type diffusion chamber; skin integrity evaluation; testosterone.

1. INTRODUCTION

Many topically administrated therapeutic agents present a limited or suboptimal efficacy due to low penetration into the skin. Solubility and partition coefficient are key aspects related to drug transport through the skin. Overall, high solubility results in high drug concentration in the donor phase (or region of application of the formulation), improving the permeation flux. The drug partitioning from the donor phase into skin layers, in turn, represents the rate-limiting step for drug flux when these molecules are characterized by a poor aqueous solubility taking into account the lipid constitution of biological membranes (Ceschel et al., 2005).

Steroidal hormones, for example, are characterized by a low aqueous solubility (Norman e Litwack, 1997), requiring the investigation of technological/formulation approaches aiming to increase the free drug concentration in the donor phase. Drug complexation (e.g. association with cyclodextrins), incorporation in vesicular or particulate systems (e.g. micro- and nanoparticles) and supersaturated systems have been often considered for this purpose (Ceschel et al., 2005).

On the other hand, the low aqueous solubility of hormones allows a drug partitioning from the donor phase into skin layers. Due to the high value of log P, these compounds bind so strongly to the tissues, particularly the more superficial layers (SC and viable epidermis), resulting in a reservoir effect and low transport rate to the dermis (Magnusson et al., 2006). This event is particularly noticed for TST after application of low concentrations of this agent on the skin (Schlupp et al., 2014).

In addition to the slow tissue diffusion because the high affinity by lipids and high molar mass, the skin presents receptors for these compounds due to the physiological action they play in that region of the body. The TST interacts directly with androgen receptors (ARs), which have been localized in most epidermis keratinocytes. In the dermis, ARs were found in approximately 10% of fibroblasts. AR expression was also found in both basal cells and sebocytes of sebaceous glands whereas it was restricted to dermal papilla cell in hair follicles (Pelletier e Ren, 2004). A modest reduction in epidermal thickness is also observed in TST replacement therapies (Kao et al., 2001).

In view of this high affinity of sex hormones by skin tissues, the inclusion of chemical absorption enhancer combinations able of providing membrane fluidification or lipid extraction is often recommended during the development of novel formulations.

These compounds may interact with both lipid and polar domains (Magnusson et al., 2006) and thus chemical absorption enhancers of both polarities should be considered.

The evaluation of percutaneous permeation of molecules is a key step when new dermal or transdermal delivery systems are purposed and different *in silico*, *ex vivo* and *in vivo* models may be considered. Although *in vivo* human assays represent the gold standard, ethical, practical, or economic reasons have limited its use. Alternatively, tissues from different animal models, particularly pig ear skin, have been selected in many permeation studies. These tissues are easier to obtain compared to human tissues, however, high experimental variability may be found. Barrier properties may also vary from human skin depending the selected animal model (Abd et al., 2016).

In addition to the inherent variability of the special animal considered, the tissue thickness and preparation method, composition of the receptor and donor fluid can also represent sources of experimental variation. All these aspects will be discussed detailly in this review article, which considered the TST as a drug model. This drug was selected based on its biopharmaceutical limitations (low solubility and reservoir effect), the various permeation studies already carried out with this compound and its wide use in hormone replacement therapies. Although TST therapy has effectively treated hypogonadism for decades, therapies simpler and more convenient to use, safer and able to mimic physiological levels are still needed (Kaminetsky e Wynia, 2015).

The main objective of this study is to provide tools so that future permeation assays can be planned more rationally considering a series of problems that may be found in each step assay. Once free drug solutions are frequently used as the control during the evaluation of the performance of new topical formulations in skin permeation studies, this review focused on the analysis of studies with free TST solutions/suspensions. The solubility of this molecule in different solvents, the importance of this physicochemical parameter in the design of permeation studies as well as aspects related to the drug quantification step are described in detail.

2. SOLUBILITY STUDIES

Prior to the cutaneous permeation assay, a study of the drug's solubility in different media should be performed. Drug should be soluble in both media used for donor and receptor phases of diffusion chambers and in solvents used to extract the drug from tissues or analytical/quantification step. If a drug is not soluble in the receptor

phase, for example, low drug partitioning into this phase with consequent low transdermal flux is obtained (permeation rate can be underestimated) and solubilizing agents or cosolvents need to be included in these situations. For this reason, many permeation guidelines provide information on the need to carry out these assays under "sink conditions" (OECD, 2004). In other words, the drug concentration should not reach values >10% of its saturation to assure that concentration in the receptor fluid does not limit the drug permeation across the tissue (Azarmi et al., 2007)

For ionizable drugs, solubility study at different pH values should be carefully performed. The ionized-non-ionized drug fraction changes with pH, which impacts not only on solubility but also on the affinity by the biological tissues.

In general, the thermodynamic solubility is evaluated via a long duration incubation (24-72 h) starting with solid materials, which is known as shake-flask method (Zhou et al., 2007). After adding an excess of solid material in given amount of solvent (above saturation), the flasks are placed on an orbital shaker with controlled temperature for 18-72 h (until the system reaches a thermodynamic equilibrium condition). Temperature close to that used in permeation assays should be preferentially selected. Finally, an aliquot of this medium is removed and then centrifuged or filtered through a 0.45 mm membrane for drug quantification.

Ethanol has been one of the most used solvents for the solubilization of drugs. It may also play a role as a skin penetration enhancer (Williams e Barry, 2012), but disruption in the SC may be observed depending on used ethanol concentration. Overall, the permeation assays reported for the TST use high concentrations of ethanol, or even the solvent pure in the donor receptor (Hewitt et al., 2020). Although permeation of lipophilic molecules is less affected by ethanol concentration than hydrophilic molecules (Horita et al., 2012), the establishment of a maximum amount of solvent is an important criterion for not having overestimated permeation values. The use of ethanol pure has been suggested to markedly alter skin permeability (Thomas e Panchagnula, 2003). The solvent evaporation is another concern regarding the use of ethanol in the donor phase. This fact leads to an increase in the TST concentration with formation of precipitates, which impairs the entry of the molecules into the SC. Thus, the evaporation rates depend on ethanol concentration, duration of the assay and temperature used.

Although the solubility of TST is higher in pure ethanol, its combination with water or buffer solutions can be considered for skin permeation studies using Franz-type diffusion cells. Determination of experimental conditions as concentration of TST in the donor chamber and the temperature used in the assay are relevant for the selection of hydroethanolic solution. An increase in ethanol concentration from 20 to 50%, for example, increases the TST solubility in approximately 31-fold. When this parameter is changed from 50 to 70 %, in the same conditions of temperature (37 ºC) and agitation time (48 h), the hormone solubility increases about 6 times (Table 1).

Propylene glycol has also been used in various percutaneous permeation studies with TST. The hormone solubility in PBS/propylene glycol (1:1) is approximately 24-fold higher than in pure PBS (Table 1). PG can also act as a chemical permeation enhancer. This agent permeates through human SC and can change the thermodynamic activity of drugs (Williams and Barry, 2012). Carrer et al., (2020) investigated its effect on the transport of molecules with different physicochemical properties. Interestingly, the permeation enhancing effect of PG was more pronounced for hydrophilic compounds. Protein denaturation (solvation the α-keratin structures of the cells) is the most probable mechanism of action, which can contribute to the barrier disruption and fluidization of intercellular lipids or intracellular expansion (Haq e Michniak-Kohn, 2018).

Table 1. Testosterone solubility in different solvents and experimental conditions.

Medium/solvent	***pH***	***Temperature***	***Incubation time***	***Solubility***	***Reference***
HEPES (10 mM + NaCl 0,154 M)	7.4	25 ºC	18 h	0.02 mg/mL	(Schwarz et al., 2017)
HEPES + HSA (4% w/v)	7.4	25 ºC	18 h	0.21 mg/L	
HEPES + β-CD (1,5% w/v)	7.4	25 ºC	18 h	0.06 mg/mL	
HEPES + HP-β-CD (4% w/v)	7.4	25 ºC	18 h	3.90 mg/mL	
HEPES + SBE7-β-CD (4% w/v)	7.4	25 ºC	18 h	3.23 mg/mL	
HEPES + HSES (1,5% w/v)	7.4	25 ºC	18 h	5.72 mg/mL	
HEPES + HTMT (1,5% w/v)	7.4	25 ºC	18 h	2.23 mg/mL	
HEPES + HTG (1,5% w/v)	7.4	25 ºC	18 h	0.07 mg/mL	
Ethanol/PG/Water (4:1:1)	-	25 ºC	12 h	26.99 mg/mL	(Leichtnam et al., 2006)
Isopropyl myristate	-	25 ºC	Several days	0.03 mg/mL	(Imai et al., 2016)
Water	-	25 ± 1 ºC	72 h	0.02 mg/mL	(Lu et al., 2013)
PBS	7.4	25 ± 1 ºC	72 h	0.02 mg/mL	
PEG 200 water solution (40%)		25 ± 1 ºC	72 h	0.32 mg/mL	
Azone®	-	25 ± 1 ºC	72 h	92.38 mg/mL	
***N*-methyl-2-pyrrolidone (NMP)**	-	25 ± 1 ºC	72 h	518.89 mg/mL	

PG	-	25 ± 1ºC	72 h	103.00 mg/mL	
Isopropyl myristate	-	25 ± 1ºC	72 h	6.83 mg/mL	
Ethanol/water (70:30)	-	37 ºC	48 h	68.31 mg/mL	(Kim et al., 2001)
Ethanol/water (70:30) +Dodecylamine 1% (w/v)	-	37 ºC	48 h	66.71 mg/mL	
Ethanol/water (70:30) + oleic acid 1% (w/v)	-	37 ºC	48 h	68.71 mg/mL	
Ethanol/water (70:30) + lauric acid 1% (w/v)	-	37 ºC	48 h	66.35 mg/mL	
Ethanol/water (70:30) + HPE-101 1% (w/v)	-	37 ºC	48 h	64.11 mg/mL	
Ethanol/water (70:30) + transcutol® 1% (w/v)	-	37 ºC	48 h	69.05 mg/mL	
PBS	-	3 ºC	24 h	0.06 mg/mL	(Mills et al., 2006)
PBS/Ethanol (50:50; w/w)	-	3 ºC	24 h	1.85 mg/mL	
PBS/ PG (50:50; w/w)	-	3 ºC	24 h	1.50 mg/mL	
PBS	-	30 ºC	24 h	0.06 mg/mL	(Mills, 2007)
PBS/Ethanol (50:50; w/w)	-	30 ºC	24 h	1.82 mg/mL	
PBS/ PG (50:50; w/w)	-	30 ºC	24 h	1.48 mg/mL	
PG	-	30 ºC	48-72 h	75.90 mg/mL	(Bonina et al., 1993)
Liquid petrolatum	-	30 ºC	48-72 h	0.43 mg/mL	
Water	-	30 ºC	48-72 h	0.03 mg/mL	
Transcutol®	-	30 ºC	48-72 h	104.00 mg/mL	
Labrasol®	-	30 ºC	48-72 h	46.10 mg/mL	
Labrafil®	-	30 ºC	48-72 h	20.90 mg/mL	
DPPG	-	30 ºC	48-72 h	13.20 mg/mL	
Water	-	32 ºC	1 or 2 weeks	0.03 mg/mL	(Binks et al., 2012)
PBS	-	32 ºC	1 or 2 weeks	0.04 mg/mL	
Isopropyl myristate	-	32 ºC	1 or 2 weeks	8.08 mg/mL	
20% hydroethanolic solution	-	20 ºC	48 h	0.25 mg/mL	(Ainbinder e Touitou, 2005)
20% hydroethanolic solution	-	37 ºC	48 h	0.34 mg/mL	
20% ethanol in PBS	-	20 ºC	48 h	0.22 mg/mL	
20% ethanol in PBS	-	37 ºC	48 h	0.30 mg/mL	
50% hydroethanolic solution	-	20 ºC	48 h	6.76 mg/mL	
50% hydroethanolic solution	-	37 ºC	48 h	10.77 mg/mL	
0.5% Brij® 98 aqueous solution	-	20 ºC	48 h	0.06 mg/mL	
0.5% Brij® 98 aqueous solution	-	37 ºC	48 h	0.08 mg/mL	
6.0 % Brij® 98 aqueous solution	-	20 ºC	48 h	0.17 mg/mL	
6.0 % Brij® 98 aqueous solution	-	37 ºC	48 h	0.19 mg/mL	
Hydrated soybean oil	-	27 ºC	-	6.00 mg/g	(Land et al., 2005)
Desiccated soybean oil	-	27 ºC	-	8.60 mg/g	
Hydrated olive oil	-	27 ºC	-	5.20 mg/g	
Desiccated olive oil	-	27 ºC	-	8.00 mg/g	
Hydrated miglyol 812 oil	-	27 ºC	-	8.90 mg/g	
Desiccated miglyol 812 oil	-	27 ºC	-	14.30 mg/g	
Ethanol	-	25 ºC	One whole day and night	209.99 mg/mL	(Araya et al., 2005)
Water	-	25 ºC	One whole day and	0.02 mg/mL	

			night		
PBS	-	30 ºC	24 h	0.07 mg/mL	
Ethanol/PBS (50:50; w/w)	-	30 ºC	24 h	6.31 mg/mL	(Mills, 2007)
PG/PBS (50:50; w/w)	-	30 ºC	24 h	5.14 mg/mL	

HSA = human serum albumin ; β-CD = β-cyclodextrin; HP-β-CD = hydroxypropyl-β-cyclodextrin; SBE7-β-CD = sulfobutyl-β-cyclodextrin; HSES = heptakis-6-sulfoethylsulfanyl6-deoxy- β-cyclodextrin; HTMT = eptakis-6-methylsulfanyl-6-deoxy-2-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)]-β-cyclodextrin; HTG = heptakis-6-thioglyceryl-6-deoxy-β-cyclodextrin; LBS = glycolysed ethoxylated C_8/C_{10} glycerides; LBF = glycolysed ethoxylated glycerides; TSC = diethylene glycol monoethyl ether; DPPG = propylene glycol dipelargonate; PBS = phosphate-buffered saline; PG = propylene glycol.

3. RECEPTOR PHASE SELECTION

The diffusion cell apparatus has been widely used for percutaneous permeation studies of drugs and it may be categorized into static or flow-through cells. In both classes, formulations are applied to the surface of a membrane, which is sandwiched between a donor and a receiver compartment of the diffusion cell (Moser et al., 2001). In static diffusion cells, samples are collected and the same volume of fresh perfusate is added at each time point (Bronaugh e Stewart, 1985). In these systems, the membrane, donor and receiver chambers may be placed either “vertically” as in the popular Franz diffusion cell or “horizontally” (Zsikó et al., 2019). In flow-through diffusion cells, a pump continuously supplies the receiver compartment with perfusate, simulating the blood flow from the dermis (Bronaugh e Stewart, 1985). When more physiologically relevant assessments of percutaneous permeation for lipophilic compounds are needed, flow-through diffusion cells should be prioritized (Clowes et al., 1994).

In static diffusion cells (most used in skin permeation studies), the volume of diffusion cell receiver chamber should be selected to guarantee detectable concentrations of the permeant in the receiver medium. In this context, the receptor phase should present a reduced volume for the evaluation of drugs with low permeation rate as TST. Likewise, the smaller the amount of TST added in the donor phase, the smaller volumes of receiver medium are recommended. The system agitation should be enough to obtain a homogenous distribution of the permeant and temperature equilibrium in this medium.

The composition of receiver phase should ensure that “sink conditions” may be achieved and barrier properties of biological tissues are preserved (Cilurzo et al., 2018; Ng et al., 2010). In an acceptable sink condition, the maximum concentration of drug in the receptor fluid in the permeation assay should not exceed 10% of its maximum solubility (EMA, 2012). It is calculated by the relationship between CS and CD (“CS”

is the saturated solubility of the compound in the medium whereas “CD” represents the concentration of compound in the bulk medium).

Isotonic saline or buffered isotonic saline (pH=7.4) are often used for highly soluble drugs to mimic the physiological environment; however, solubilizer agents need to be included when poorly water-soluble drugs are considered (Finnin et al., 2012). Azone® and *N*-methyl-2-pyrrolidone, for example, increased the hormone solubility from 0.02 mg/mL (in water) to 92.29 mg/mL and 518.89 mg/mL, respectively (Table 1). Although the solubilization of TST by these agents allows sink conditions to be achieved, the impact on the skin structure discourages their application (Fang et al., 2003). Thus, aqueous solutions containing less aggressive agents such as ethanolic solutions, bovine serum albumin or propylene glycol should be alternatively considered for the composition of receptor fluid (Table 2). Sodium azide was also added to the receptor solution in some studies with an experimental time of 24 h (Table 2). This compound acts as a preservative and is therefore recommended for long-term permeation assays (Bartosova e Bajgar, 2012).

4. EFFECT OF DRUG CONCENTRATION FROM DONOR PHASE

According to Fick's 1st law (Equation 1), the permeation flux of a drug is proportional to its concentration in the vehicle (or thermodynamic activity, A_v), occurring in favor of a concentration gradient. High drug solubilization leads to a high thermodynamic activity in the donor phase, improving its permeation flux through the SC (Ceschel et al., 2005).

$$J = \frac{K.C_0.D}{L} = \frac{\gamma_v.C_0.D}{\gamma_s.L} = \frac{A_v.D}{\gamma_s.L} \quad \text{(Equation 1)}$$

Where: K, C_0, D, γ_v and γ_s represent the partition coefficient, initial concentration in the formulation, diffusion coefficient, activity coefficient in the formulation and that from the skin barrier, respectively. L is the tissue thickness (Ishii et al., 2010).

The maximum permeation flux (J_{max}), in turn, is achieved at the maximum solubility (S_s) of a solute in the SC (Couto et al., 2014), which also corresponds when solute solubility in vehicle achieves its saturation condition.

$$J_{max} = \frac{D.S_s}{L} \quad \text{(Equation 2)}$$

In general, the permeation profile of TST through the skin follows a Fickian diffusion given the absorbed drug fraction or rate in specific period is constant. When the diffusion of the TST in the skin is analyzed, its transfer rate to the SC is faster whereas a slower rate from the dermis to the systemic route is showed. As already discussed, this fact is attributed to reservoir effect of TST in tissue. The greater the interaction or partitioning of the drug with the tissue, the slower the diffusion rate.

The fluid composition of the donor phase appears to impact on the permeation rate more significantly than the TST concentration (Table 2). In a study that considered 0.22, 6.31 and 5.14 mM of TST in only PBS, ethanol/PBS and propylene glycol/PBS in the donor phase of diffusion cells, permeation coefficient (K_p) values from equine skin were 6.82, 1.59 and 2.04 x 10^{-3} cm/h, respectively (Mills, 2007). Although the donor phase containing only PBS had a lower drug concentration, it provided a higher K_p value. These findings may be associated with solubility differences of TST in the donor phase, which affect the supersaturation degree (greater in aqueous solutions). In another study considering a similar donor phase composition (solubilizer type and ratio as well as TST concentration) and canine skin as membrane model, donor solutions without solubilizer agent and with lower TST concentration also provided higher K_p values (Mills et al., 2006). In the same way, in a permeation study carried out with different concentrations of ethanol in water (20, 40, 50, 60, 70, 80 and 100 %) and TST (0.45, 8.53, 18.52, 38.95, 68.32, 140.01 and 33.04 mg mL^{-1}), lower K_p values through rat skin were found for increasing amounts of ethanol and TST (Kim et al., 2001). In these analyzed studies, the inclusion of propylene glycol seems to be more advantageous than ethanol in increasing the cutaneous transport of TST.

In permeation studies, authors commonly mention that an increased thermodynamic activity provided by a state of supersaturation results in higher permeation rate (Schwarb et al., 1999), however, it is necessary to be careful with this assumption. In a study comparing the permeation rate of TST from saturated solution and suspensions presenting different supersaturation degrees (1.4, 2.1 and 2.6), only the suspension with a supersaturation degree of 1.4 provided a higher flux permeation than control. For the other conditions, a permeation flux similar to the saturated solution was found because drug precipitation phenomena would be observed, reducing the free drug amount available to be absorbed (M.-L. Leichtnam et al., 2006) Considering these findings, it is important to consider the inclusion of anti-precipitating agents in tested

medium so that the advantages resulting from the increase in the degree of supersaturation can be observed. This may also explain why membrane-based systems (e.g., polymeric films or transdermal patches) can provide higher rates of cutaneous permeation compared to the solution or other liquid systems in specific situations.

5. IMPACT OF TISSUE PREPARATION ON DRUG PERMEATION

Permeation assays may be performed by using epidermal membranes, dermatomed skin, or full-thickness skin. The use of epidermis provides a greater correlation with *in vivo* situation (in humans). The presence of dermis in dermatomed skin and full thickness skin acts as an unreal barrier since continuous blood flow occurs within watery dermis only in *in vivo* environment. Although the use of the epidermis provides more realistic information on drug transport (Barbero e Frasch, 2016), the tissue preparation is more difficult and susceptible to structural damages. Currently available methods to separate the epidermis from the dermis use heating or chemical treatment (e.g. treatment with salts, detergents, enzymes) (Abd et al., 2016), which can affect both tissue integrity and skin metabolic activity. The tissue treatment with trypsin, for example, may lead to a SC more heterogeneous, contributing to the partitioning of more polar solutes (Magnusson et al., 2006). Another disadvantage is that hair follicles may be damaged during the tissue separation process, leading to drug leakage (Barbero e Frasch, 2016).

The transappendageal route is preferentially used to transport hydrophilic drug, high molecular weight compounds and drug delivery systems as nanoparticles (Knorr et al., 2009); however, Hueber et al. (1992) has also demonstrated its contribution in transport of TST (via sebaceous glands). In another study performed by the same research team (Hueber et al., 1994), the absorption of TST from the human normal skin was approximately 2.4-fold higher than in human scar skin (without skin appendages) at the end of 8 h. These findings showed the importance of preserving the appendages of the skin in permeation studies.

Transfollicular drug delivery may be evaluated by comparing different body regions, use of specific animal models, artificial introduction of hair follicles in skin equivalents and/or blockage of hair follicles compared to untreated skin (Knor et al., 2009). Porcine skin is regarded as the most representative animal model to determine the contribution of the follicular route in drug transport due to the similarity to human

skin. In fact, porcine and human skin present about 20-30 follicles per cm^2 of skin area, and a hair density of 11-25 hairs/cm^2 with a diameter of 58-97 μm. Animal models such as rat and mouse skin exhibit much more follicles, with smaller diameters when compared to human skin (Lauterbach e Müller-Goymann, 2015). Follicle-free skin models as EpidermFT™ (for comparison with human skin presenting follicles) and *in vitro* models of human fibroblasts or keratinocytes with hair follicles may represent alternatives to animal use in these studies (Krugluger et al., 2005; Michel et al., 1999).

"Full-thickness skin" is prepared by removal of connective tissue and subcutaneous fat and its thickness may be reduced with a dermatome. This procedure reduces experimental variability (Abd et al., 2016). Although dermatomed and full-thickness skin present an additional barrier that is not found *in vivo*, particularly for lipophilic drugs, the tissue integrity is preserved (Barbero e Frasch, 2016). As a result, the absorption rate of drugs could be underestimated. The presence of this additional layer represents a situation analogous to vasoconstriction (Abd et al., 2016).

Interestingly, a study testing only the dermis layer as membrane model and TST was also performed (Kretsos et al., 2008). The permeation coefficient value found from this study showed to be higher than other studies performed with full-thickness or dermatomed skin as well as only with human epidermis (Baert et al., 2012; Guth et al., 2015; Hewitt et al., 2020; J R Heylings et al., 2018; Kretsos et al., 2008; Netzlaff et al., 2006; Qvist et al., 2000; Schreiber et al., 2005; Veryser et al., 2015), confirming that both SC and viable epidermis play key barrier properties.

A significant difference in permeation flux of TST through human dermatomed skin (most of the dermis removed) and full-thickness skin was observed, with a TST flux of approximately 10-fold higher in the thinner skin (Wilkinson et al., 2006). Thus, membrane thickness should be a critical variable for TST permeation across human skin. The intra- and inter-laboratory study between 10 laboratories found higher flux rates of TST using skin samples presenting thickness between 300 and 500 μm (2.82 to 5.39 $\mu g.cm^{-2}.h^{-1}$) compared to skin samples with thickness between 700 and 900 μm (0.40 to 0.80 $\mu g.cm^{-2}.h^{-1}$) (van de Sandt et al., 2004). This finding is consistent with the lipophilic nature of TST, which difficult its transport through the dermis. Therefore, permeation results obtained for lipophilic agents after the transport in full-thickness or dermatomed tissue preparations should be carefully analyzed (Magnusson et al., 2006).

The use of epidermal membranes, in turn, may overestimate the drug absorption in humans because of insufficient barrier function. The use of cultured and reconstructed human skin models (e.g. constructed from keratinocytes) is not recommended for the determination of dermal penetration as these models have not been validated for dermal absorption studies and there are reports that their barrier properties are not comparable with those of 'natural origin' skin (SCCS, 2010; WHO, 2006).

The OECD recommends the use of dermatomed skin with a thickness between 200 and 400 μm for harmonization of *ex vivo* studies with human skin. In these range of thickness, the membranes tend to have significantly lower levels of residual material than full-thickness preparations. For permeation assays with TST (Table 1), dermatomed and full-thickness skin models appears in most of these studies. The use of membranes with 400 μm of thickness was prevalent among the studies with human skin, even though, different values of permeated drug and rate flux were showed. Higher permeability coefficient values (K_p) have been reported with the use of only epidermis compared to dermatomed skin as already mentioned. Correlations can be established by regarding studies performed in similar experimental conditions (donor and receptor fluid). Netzlaff et al. (2006) and Schreiber et al. (2005), for instance, found K_p values of 9.4 x 10^{-4} and 8.3 x 10^{-4} cm.h^{-1}, respectively, with human epidermis separated by heat. In contrast, lower K_p values, 4.5 x 10^{-4} and 3.92 x 10^{-4} cm.h^{-1}, were showed by Baert et al. (2012) and Veryser et al. (2015), respectively, using skin samples with a thickness of 400 μm. In fact, the tissue preparation has a significant effect on the TST permeation. The studies showed an increase of about 2-fold in permeation rate of the hormone without dermis layer.

The tissue storage conditions also impact on drug permeation. As the skin preparation step is laborious, the tissue is commonly frozen for a specific period before the use in the permeation assays. The freezing does not affect the skin barrier integrity and it is a suitable procedure to measure the passive permeation of drugs (Barbero e Frasch, 2016). One the other hand, it is worth mentioning that frozen tissue is not appropriated to investigate the metabolic activity of drugs (Fahmy et al., 1993).

In general, the permeation assays with TST were performed using similar storage conditions. The tissues were frozen at -20 ºC in all studies with human skin, which is according with regulatory agencies guidelines (OECD, EU Scientific

Committee on Consumer Products, US Environmental Protection Agency, International Programme on Chemical Safety). Tissue preparation for permeation studies was performed prior to freezing. The human skin storage time, in turn, often ranged from 3 (Baert et al., 2012; Hewitt et al., 2020) or 6 months (Schreiber et al., 2005; Veryser et al., 2015). A consensus among regulatory agencies regarding the freezing time of tissues prior the use has not yet been reached. For example, the International Programme on Chemical Safety (IPCS) states that human skin can be stored to one year, whereas the US Environmental Protection Agency (EPA) allows the storage for up to 3 months. The skin thawing should also be standardized in assays since the use of frozen skin without hydration provides different permeation rate values when compared to fresh tissue. Thus, the tissues should be appropriately rehydrated before use (Swarbrick et al., 1982).

6. SELECTION OF ANIMAL MODELS OR MEMBRANE TYPE

Human skin is the preferred membrane model to predict the *ex vivo* permeation of compounds. Excised skin is commonly obtained from autopsies (cadaver skin) or plastic surgery. Overall, the permeation assays of TST were performed with tissues from abdomen and breast. Several animal models have been alternatively considered to human skin, including pig, mouse, rat, guinea pig and rabbit skin. Porcine skin has been widely used given its histological similarity to human skin. It presents a comparable SC thickness, which varies from 21 to 26 μm, and similar hair-follicle density (20 vs. 14-32/cm^2 for porcine ear skin and human forehead skin, respectively) (Jacobi et al., 2007). Porcine SC lipids are organized as a hexagonal lattice whereas human SC lipids are arranged in the denser orthorhombic lattice (Caussin et al., 2008).

The easy availability and relatively low cost make the group of rodents (mice, rat, and guinea pigs) widely used in *ex vivo* percutaneous permeation studies. Although rat skin is most structurally similar to human skin among the rodents, rat dermis is thicker than human and does not present a defined limit between papillary and reticular dermis. In addition, rats have no subcutaneous fatty tissue or subcutaneous muscle tissue (McFarlane et al., 1965). In terms of permeability, significant differences between rat and human skin have been found for various compounds presenting different physicochemical properties (Abd et al., 2016). *Ex vivo* permeation studies with saturated and supersaturated solutions of TST applied in rat skin have been performed (Kim et al.,

1999; Leichtnam et al., 2006). Overall, higher values of flux and permeation coefficient were found with rat skin compared with human skin (Table 1). Nevertheless, the rat is also often used for *in vivo* studies because the extensive pharmacokinetics/ pharmacodynamics data that may be obtained with this species.

Netzlaff et al. (2006) performed *ex vivo* permeation studies with TST and skin models from different species. The permeation coefficient values of TST through bovine udder, human and pig skin were 5.42, 2.31 and 1.29 (x 10^{-7}) $cm.s^{-1}$, respectively. Differences in skin composition explain these findings. Human and pig skin are characterized by a much higher lipid fraction (triglycerides and free fatty acids) than udder skin, which increase the affinity of TST by the tissue. Although these tissues significantly differ in relation to the number of follicles per tissue area (6, 30-36 and 207-338 $mm.cm^{-2}$ for human, pig and bovine udder skin, respectively), this permeation route is not used for lipophilic molecules such as TST. The epidermal thickness also varied with the animal species (57-82, 40 and 54-92 μm for human, pig and bovine udder skin, respectively), but this parameter would present a marginal contribution on permeation rate of TST.

TST has been used as lipophilic molecule model in several percutaneous permeation studies. When the different studies are compared (Table 2), mice, amphibian and equine skin seem to be more permeable to TST than human or porcine skin (K_p values increased from 10 to 100 times). In fact, amphibian skin, for example, presents only 1-2 cell layers of stratum corneum with no intercellular lipid layers, offering less resistance to TST transport (Lillywhite, 2006). In the same way, mice also have a stratum corneum that is thinner than humans (5 vs. 10-20 μm) (Wei et al., 2017), which may explain the greater permeation rate.

As amphibian skin is much more permeable than other vertebrates and more sensitive to environmental contaminants, this skin model has not only been considered to assess the absorption of molecules but also as an indicator of the relative health of an ecosystem (Llewelyn et al., 2019). Kaufmann and Dohmen (2016) performed permeation assays with TST in the African clawed frog skin (X. laevis, wild type). The permeation coefficient values ranged from 1.3 to 3.0 x 10^{-3} $cm.h^{-1}$. Regarding the same composition of donor and receptor fluid, Baert et al. (2012) evaluated the TST permeation across human skin and found a permeation coefficient of 4.5 x 10^{-4} $cm.h^{-1}$.

Therefore, the permeation of TST through amphibian skin was 2.9 to 6.7-fold higher than human skin.

In vitro permeation studies in equines and canines have also been performed (Mills, 2007; Mills et al., 2006) since the skin is an administration route used for the TST in these animals. The main clinical use of TST in horses aims to increase libido and treat hypogonadism (Snow, 1993). Its application to improve the performance of animals in competitions is illegal (Houghton e Maynard, 2010). In dogs, the hormone is particularly used for treating TST-responsive urinary incontinence and for suppression of oestrus in racing greyhounds (Blythe et al., 1994; Plumb, 2002).

Synthetic membranes are structurally simpler and demonstrate superior permeation data reproducibility since *in vivo* variables as skin age, race, sex and anatomical site are eliminated. In contrast, they do not exhibit the lipid perturbation effects undergone by biological samples (Ng et al., 2010). For the TST, all synthetic silicone-based membranes demonstrated lower barrier properties and thus greater permeation compared to biological tissues. The fact that synthetic membranes do not have receptors for TST, and present greater permeability may raise the hypothesis of a probable relationship between physiological receptors and the reservoir effect observed for this hormone; however, further investigations should be performed.

Reconstructed skin models are interesting substitutes for human and animal skin because they overcome the problems of availability of human skin and the ethical issues of using animals. On the other hand, the OECD has not yet approved the use of these models for skin absorption assays (only for *in vitro* skin irritation assays). Therefore, more studies are needed to validate the models for this purpose. In addition, reconstructed skin is usually more permeable than human skin. The low barrier property of reconstructed skin was demonstrated by Schreiber et al. (2005). The authors founded higher permeation coefficient values for TST using reconstructed epidermis (K_p= 176.4 x 10^{-5} and 777.6 x 10^{-5} cm.h^{-1}) compared to human epidermis (K_p= 7.6 x 10^{-5} cm.h^{-1}) and porcine skin (K_p= 31.7 x 10^{-5} cm.h^{-1}). The assays with porcine skin and human epidermis were performed in 24 h whereas the use of reconstructed skin reduced this time to 6 h. The short lag time and high flux values are associated with the reduction of experimental time. For this reason, the reconstructed skin model has been considered advantageous for routine and screening assays, particularly when several compounds or topical formulations should be analyzed at the same time.

The efforts to improve the barrier function of these models is constant. Simard et al. (2019), for instance, evaluated the effect of different lipids on skin barrier properties by considering a 3D reconstructed skin model. A slower permeation rate of TST was found after supplementation with alpha-linolenic acid. On the other hand, the supplementation with linoleic acid did not affect the drug absorption. Thus, a proper composition and proportion of fatty acids in culture media should be considered to achieve similar barrier properties to human skin.

Table 2. Experimental conditions used in skin permeation studies with testosterone.

Animal model	Thickness of tissue	Section of tissue	Tissue preparation	Composition of donor chamber	Volume of donor solution	Drug concentration in the donor chamber	Composition of receptor chamber	Volume of receptor medium	Experimental total time	Permeation parameters (lag time, permeation coefficient and flux)	Retained drug amount or information on TST retention	Protein precipitation solvent (Y or N) / freezing samples? (Y or N)	Quantification method / Injected amount (HPLC)	Area for permeation	Reference
Human cadaver skin	300 µm	Dermis	Dermatomed tissue, following by heating at 55°C for 2min	TST solubilized in ethanol	100 µL	50Ci/mL of ^{3}H-TST	PBS, PBS + 2% BSA or PBS + 2% HSA	6 mL	-	3.3 x 10^{-3} cm/h	NF	N N	Liquid scintillation counting	1.77 cm^2	(Kretsos et al., 2008)
Neonatal piglet skin	-	Epidermis and dermis	Surgical scissors	TST (4% w/v) solubilized in ethanol 95% (V/V)	5 µL	4% of TST	Ethanol 20% (v/v) and sodium azide 0,1% (m/v) in water	NF	24 h	0.059 µg/cm².h	NF	N N	HPLC 20 µL	0.79 cm^2	(Nicolazzo et al., 2005)
Neonatal piglet skin	-	Epidermis and dermis	Surgical scissors	TST (4% w/v) solubilized in ethanol 95% (V/V) and oleic acid 5% (m/V)	5 µL	4% of TST	Ethanol 20% (v/v) and sodium azide 0,1% (m/v) in water	NF	24 h	0.186 µg/cm².h	NF	N N	HPLC 20 µL	0.79 cm^2	
Neonatal piglet skin	-	Epidermis and dermis	Surgical scissors	TST (4% w/v) solubilized in ethanol 95% (V/V), followed by application of 400 µL of PG 20%	5 µL	4% of TST	Ethanol 20% (v/v) and sodium azide 0,1% (m/v) in water	NF	24 h	0.515 µg/cm².h	NF	N N	HPLC 20 µL	0.79 cm^2	
Nude mouse skin	-	Full-thickness skin	NF	TST in 50% aqueous ethanol solution	3.1 mL	27 mg/mL (TST was at 71% saturation)	Hydroalcoholic solution (50% ethanol)	3.1 mL	25 h	2.5 x 10^{-3} cm/h	NF	N N	Scintillation counting	NF	(Kaplun-Frischoff e Touitou, 1997)
Nude mouse skin	-	Full-thickness skin	NF	Inclusion of a chemical absorption enhancer Eutectic mixture of menthol:TST (4:1) in 50% aqueous ethanol solution	3.1 mL	76.8 mg/mL	Hydroalcoholic solution (50% ethanol)	3.1 mL	25 h	6.6 x 10^{-3} cm/h	NF	N N	Scintillation counting	NF	
Silastic membrane (silicone membrane)	-	-	NF	TST in 50% aqueous ethanol solution	3.1 mL	27 mg/mL (TST was at 71% saturation)	Hydroalcoholic solution (50% ethanol)	3.1 mL	25 h	9.95 x 10^{-3} cm/h	NF	N N	Scintillation counting	NF	
Silastic membrane (silicone membrane)	-	-	NF	Inclusion of a chemical absorption enhancer Eutectic mixture of menthol:TST (4:1) in 50% aqueous ethanol solution	3.1 mL	76.8 mg/mL	Hydroalcoholic solution (50% ethanol)	3.1 mL	25 h	10.46 x 10^{-3} cm/h	NF	N N	Scintillation counting	NF	
Rat skin	-	Epidermis+dermis	Surgical materials	Ethanol/water (70/30)	3 mL	Saturated solution of TST	Saline solution containing 40% (v/v) polyethylene glycol 400	12 mL	24 h	0.04 x 10^{-3} cm/h (lag time = 2.88 h)	NF	N Y(-20°C)	HPLC 20 µL	2.14 cm^2	(KIM et al., 2000)
Rat skin	-	Epidermis+dermis	Surgical materials	Ethanol/water (70/30) plus 1% (m/V) transcutol	3 mL	Saturated solution of TST	Saline solution containing 40% (v/v) polyethylene glycol 400	12 mL	24 h	0.04 x 10^{-3} cm/h (lag time = 1.96 h)	NF	N Y(-20°C)	HPLC 20 µL	2.14 cm^2	
Rat skin	-	Epidermis+dermis	Surgical materials	Ethanol/water (70/30) plus 1% (m/V) lauric acid	3 mL	Saturated solution of TST	Saline solution containing 40% (v/v) polyethylene glycol 400	12 mL	24 h	0.09 x 10^{-3} cm/h (lag time = 5.5 h)	NF	N Y(-20°C)	HPLC 20 µL	2.14 cm^2	

Rat skin	-	Epidermis+dermis	Surgical materials	Ethanol/water (70/30) plus 1% (m/V) oleic acid	3 mL	Saturated solution of TST	Saline solution containing 40% (v/v) polyethylene glycol 400	12 mL	24 h	0.20 x 10^{-3} cm/h (lag time = 7.04 h)	NF	N Y(-20°C)	HPLC 20 µL	2.14 cm^2	
Rat skin	-	Epidermis+dermis	Surgical materials	Ethanol/water (70/30) plus 1% (m/V) dodecylamine	3 mL	Saturated solution of TST	Saline solution containing 40% (v/v) polyethylene glycol 400	12 mL	24 h	0.72 x 10^{-3} cm/h (lag time = 7.09 h)	NF	N Y(-20°C)	HPLC 20 µL	2.14 cm^2	
Human skin	400 µm	Epidermis+dermis	Dermatomed tissue	[^{14}C]-TST was dissolved in 40% ethanol in water	10 µL/cm^2	10 µL/cm^2 (from a solution with 1 mg/mL of TST)	Physiological saline + 5% bovine serum albumine + 0.1% sodium azide	4.5 mL	24h	0.005 µg/cm^2.h	Stratum corneum (0.16%); remaining epidermis (0.41%); dermis (0.26%)	N N	Scintillation Tissue digestion	2.54cm^2	(J R Heylings et al., 2018)
Pig skin	400 µm	Epidermis+dermis	Dermatomed tissued	[^{14}C]-TST was dissolved in 40% ethanol in water	10 µL/cm^2	10 µL/cm^2 (from a solution with 1 mg/mL of TST)	Physiological saline + 5% bovine serum albumine + 0.1% sodium azide	4.5 mL	24h	0.055 µg/cm^2.h	Stratum corneum (0.23%); remaining epidermis (0.89%); dermis (1.17%)	N N	Scintillation Tissue digestion	2.54cm^2	
Human skin	400 µm	Epidermis+dermis	Dermatomed tissue	Ethanol/water 50/50 (V/V)	500 µL	32 mM	PBS + 5% (m/V) bovine serum albumin	5 mL	24 h	2.08 to 6.79 x 10^{-4} cm/h (range)	NF	Y (acetonitrile) N	HPLC Limit of quantification = 0.17 µg/mL	0.64 cm^2	(Baert et al., 2012)
Equine Skin	-	Epidermis+dermis	Surgical materials	PBS	1 mL	Saturated TST solution (0.22 mmol/L)	PBS + 4% (m/V) bovine serum albumin	3.5 mL	24h	6.82 x 10^{-3} cm/h	PBS > EtOH = PG	N N	Liquid Scintillation Analyzer	-	(Mills, 2007)
Equine Skin	-	Epidermis+dermis	Surgical materials	Ethanol/PBS 50/50 (m/m)	1 mL	Saturated TST solution (6.31 mmol/L)	PBS + 4% (m/V) bovine serum albumin	3.5 mL	24h	1.59 x 10^{-3} cm/h	PBS > EtOH = PG	N N	Liquid Scintillation Analyzer	-	
Equine Skin	-	Epidermis+dermis	Surgical materials	Propylene glycol (PG)/PBS 50/50 (m/m)	1 mL	Saturated TST solution (5.14 mmol/L)	PBS + 4% (m/V) bovine serum albumin	3.5 mL	24h	2.04 x 10^{-3} cm/h	PBS > EtOH = PG	N N	Liquid Scintillation Analyzer	-	
Human skin	-	Stratum corneum + epidermis	Forceps	TST solubilized in 2% of Igepal®	0.5 mL	40 µg/mL	Phosphate buffered saline (PBS)	12.1 mL	30 h	2.31 x 10^{-7} cm/s	-	NF NF	HPLC 50 µL	1.767 cm^2	(Netzlaff et al., 2006)
Porcine skin	1000 µm	Epidermis+dermis	Dermatomed tissue	TST solubilized in 2% of Igepal®	0.5 mL	40 µg/mL	Phosphate buffered saline (PBS)	12.1 mL	30 h	1.29 x 10^{-7} cm/s	-	NF NF	HPLC 50 µL	1.767 cm^2	
Bovine skin	1000 µm	Epidermis+dermis	Dermatomed tissue	TST solubilized in 2% of Igepal®	0.5 mL	40 µg/mL	Phosphate buffered saline (PBS)	12.1 mL	30 h	5.42 x 10^{-7} cm/s	-	NF NF	HPLC 50 µL	1.767 cm^2	
Canine skin (thorax)	-	Epidermis+dermis	Forceps and scissors	Phosphate buffered saline (PBS)	1 mL	Saturated solution (0.21 mmol/L)	PBS pH 7.4 + 4% bovine serum albumin (BSA)	3.5 mL	24h	5.53 x 10^{-4} cm/h	Neck > groin > thorax PBS>PG>EtOH	N N	Scintillation Radiolabeled (14C) TST	-	(Mills et al., 2006)
Canine skin (neck)	-	Epidermis+dermis	Forceps and scissors	Phosphate buffered saline (PBS)	1 mL	Saturated solution (0.21 mmol/L)	PBS pH 7.4 +4% BSA	3.5 mL	24h	4.32 x 10^{-4} cm/h		N N	Scintillation Radiolabeled (14C) TST	-	
Canine skin (groin)	-	Epidermis+dermis	Forceps and scissors	Phosphate buffered saline (PBS)	1 mL	Saturated solution (0.21 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	3.85 x 10^{-4} cm/h		N N	Scintillation Radiolabeled (14C) TST	-	
Canine skin (thorax)	-	Epidermis+dermis	Forceps and scissors	Ethanol/PBS 50/50 (w/w)	1 mL	Saturated solution (6.26 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	1.12 x 10^{-4} cm/h	Neck = thorax > groin PBS>PG>EtOH	N N	Scintillation Radiolabeled (14C) TST	-	
Canine skin	-	Epidermis+dermis	Forceps and	Ethanol/PBS 50/50 (w/w)	1 mL	Saturated	PBS pH 7.4 + 4%	3.5 mL	24h	1.21 x 10^{-4} cm/h		N	Scintillation	-	

(neck)			scissors			solution (6.26 mmol/L)	BSA					N	Radiolabeled (14C) TST		
Canine skin (groin)	-	Epidermis+dermis	Forceps and scissors	Ethanol/PBS 50/50 (w/w)	1 mL	Saturated solution (6.26 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	0.73 x 10^{-4} cm/h		N N	Scintillation Radiolabeled (14C) TST	-	
Canine skin (thorax)	-	Epidermis+dermis	Forceps and scissors	Propylene glycol/PBS 50/50 (w/w)	1 mL	Saturated solution (5.21 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	1.63 x 10^{-4} cm/h	Neck = thorax > groin PBS>PG>EtOH	N N	Scintillation Radiolabeled (14C) TST	-	
Canine skin (neck)	-	Epidermis+dermis	Forceps and scissors	Propylene glycol/PBS 50/50 (w/w)	1 mL	Saturated solution (5.21 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	3.14 x 10^{-4} cm/h		N N	Scintillation Radiolabeled (14C) TST	-	
Canine skin (groin)	-	Epidermis+dermis	Forceps and scissors	Propylene glycol/PBS 50/50 (w/w)	1 mL	Saturated solution (5.21 mmol/L)	PBS pH 7.4 + 4% BSA	3.5 mL	24h	0.81 x 10^{-4} cm/h		N N	Scintillation Radiolabeled (14C) TST	-	
Human skin	< 1000 µm	Epidermis Full-thickness skin	Forceps and scissors	PBS + 2% Igepal®	-	0.004%	PBS	12 mL	24h	9.4 x 10^{-4} cm/h (lag time = 0.03 h)	NF	N N	HPLC	1.768 cm^2	
Reconstructed epiderm EpiDerm™	-	Epidermis	Purchased by Laboratoire MatTek Corp.	PBS + 2% Igepal®	-	0.004%	PBS	12 mL	24h	122.4 x 10^{-4} cm/h (lag time = 0 h)	NF	N N	HPLC	1.768 cm^2	(Schreiber et al., 2005)
Reconstructed epiderm SkinEthic®	-	Epidermis	Purchased by Laboratoire SkinEthic	PBS + 2% Igepal®	-	0.004%	PBS	12 mL	24h	212.4 x 10^{-4} cm/h (lag time = 0.01 h)	NF	N N	HPLC	1.768 cm^2	
Porcine Skin	< 1000 µm	Epidermis	Forceps and scissors	PBS + 2% Igepal®	-	0.004%	PBS	12 mL	24h	11.5 x 10^{-4} cm/h (lag time = 7.63 h)	NF	N N	HPLC	1.768 cm^2	
Rat skin	-	Full-thickness skin	Dermatomed skin	EtOH/propylene glycol /water (4:1:1)	250 µL	Saturated solution	PBS	Perfusion (0.6 mL/min)	6 h	2.1 µg/cm^2.h	NF	N N	HPLC	0.785 cm^2	
Rat skin	-	Full-thickness skin	Dermatomed skin	EtOH/propylene glycol /water (4:1:1)	250 µL	Supersaturated solution (supersaturation degree = 1.4)	PBS	Perfusion (0.6 mL/min)	6 h	3.1 µg/cm^2.h	NF	N N	HPLC	0.785 cm^2	
Rat skin	-	Full-thickness skin	Dermatomed skin	EtOH/propylene glycol /water (4:1:1)	250 µL	Saturated solution (supersaturation degree = 2.1)	PBS	Perfusion (0.6 mL/min)	6 h	2.2 µg/cm^2.h (drug crystallization)	NF	N N	HPLC	0.785 cm^2	(M.-L. Leichtnam et al., 2006)
Rat skin	-	Full-thickness skin	Dermatomed skin	EtOH/propylene glycol /water (4:1:1)	250 µL	Saturated solution (supersaturation degree = 2.6)	PBS	Perfusion (0.6 mL/min)	6 h	2.2 µg/cm^2.h (drug crystallization)	NF	N N	HPLC	0.785 cm^2	
Mice skin	-	Full-thickness skin	NF	TST solubilized in isopropyl myristate (1/99)	1 g	1%	PBS	7-7.3 mL	10 h	2.0 µg/cm^2.h (lag time<0.5h)	NF	N	HPLC	1.74 cm^2	
Mice skin	-	Full-thickness skin	NF	TST in isopropyl myristate/lecithin (1/69/30)	1 g	1%	PBS	7-7.3 mL	10 h	2.6 µg/cm^2.h (lag time<2h)	NF	N	HPLC	1.74 cm^2	(Imai et al., 2016)
Mice skin	-	Full-thickness skin	NF	TST in isopropyl myristate/lecithin/water (1/65.6/30/3.4)	1 g	1%	PBS	7-7.3 mL	10 h	3.98 µg/cm^2.h (lag time=1.06h)	NF	N	HPLC	1.74 cm^2	

Mice skin	-	Full-thickness skin	NF	TST in water/lecithin/D-ribose (1/62.8/30/6.2)	1 g	1%	PBS	7-7.3 mL	10 h	2.69 μg/cm^2.h (lag time=0.77h)	NF	N	HPLC	1.74 cm^2	
Mice skin	-	Full-thickness skin	NF	TST in lecithin/D-ribose/ tetraglycerol (1/30/55.6/13.4)	1 g	1%	PBS	7-7.3 mL	10 h	2.35 μg/cm^2.h (lag time=1.02h)	NF	N	HPLC	1.74 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (20:80)	3 mL	Saturated solution of TST (0.45 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	1.8 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 μL	2.01 cm^2	(Kim et al., 2001)
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (40:60)	3 mL	Saturated solution of TST (8.53 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	0.13 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 μL	2.01 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (50:50)	3 mL	Saturated solution of TST (18.52 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	0.07 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 μL	2.01 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (60:40)	3 mL	Saturated solution of TST (38.95 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	0.05 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 μL	2.01 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (70:30)	3 mL	Saturated solution of TST (68.32 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	0.04 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 μL	2.01 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol/water (80:20)	3 mL	Saturated solution of TST (140.01 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	0.02 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 μL	2.01 cm^2	
Rat Skin	-	Full-thickness skin	Surgical materials	Ethanol 100%	3 mL	Saturated solution of TST (334.03 mg/mL)	Saline solution containing 40% (V/V) polyethylene glycol 400	12 mL	24 h	0.00 x 10^{-3} cm/h	NF	N Y (-20°C)	HPLC 20 μL	2.01 cm^2	
Mice skin	-	Epidermis+dermis	Surgical materials	TST solubilized in ethanol with and without chemical absorption enhancer (Azone®, isopropyl myristate, *N*-methyl-2-pyrrolidone and propylene glycol)	100 μL	5% (m/V)	40% PEG 200	7 mL	24 h	Azone>IPM>PG>NMP	NF	N N	HPLC	3.14 cm^2	(Lu et al., 2013)
Amphibian skin (fresh dorsal)	-	Full-thickness skin	Surgical materials	TST dissolved in ethanol/water 1/1 (v/v)	10μL/cm²	40 μg/cm² (dose solution 4 mg/mL)	5% BSA in amphibian Ringer's solution	12.5 mL	8h	J = 5.3 μg/cm².h P = 1.3 x 10^{-3} cm/h	NF	N N	Liquid scintillation counting	1.85 cm²	(Kaufmann e Dohmen, 2016)

Amphibian skin (fresh ventral tissue)	-	Full-thickness skin	Surgical materials	TST dissolved in ethanol/water 1/1 (v/v)	10µL/cm²	40 µg/cm² (dose solution 4 mg/mL)	5% BSA in amphibian Ringer's solution	12.5 mL	8h	J = 12.0 µg/cm².h P = 3.0 x 10^{-3} cm/h	NF	N N	Liquid scintillation counting	1.85 cm²	
Amphibian skin (frozen dorsal tissue)	-	Full-thickness skin	Surgical materials	TST dissolved in ethanol/water 1/1 (v/v)	10µL/cm²	40 µg/cm² (dose solution 4 mg/mL)	5% BSA in amphibian Ringer's solution	12.5 mL	8h	J = 7.4 µg/cm².h P = 1.9 x 10^{-3} cm/h	NF	N N	Liquid scintillation counting	1.85 cm²	
Amphibian skin (frozen ventral tissue)	-	Full-thickness skin	Surgical materials	TST dissolved in ethanol/water 1/1 (v/v)	10µL/cm²	40 µg/cm² (dose solution 4 mg/mL)	5% BSA in amphibian Ringer's solution	12.5 mL	8h	J = 11.4 µg/cm².h P = 2.9 x 10^{-3} cm/h	NF	N N	Liquid scintillation counting	1.85 cm²	
Gottingen Minipigs (1.5 months)	765 µm	Epidermis+dermis	Dermatomed skin	20% ethanol in 0.05 M phosphate buffer (pH=7.4)	NF	0.1 mg/ml	0.05 M phosphate buffer	12.1 mL	28 h	J = 0.223 µg/cm².h Lag time: 3 to 5 h	NF	N N	Liquid scintillation counting	1.77 cm²	(Qvist et al., 2000)
Gottingen Minipigs (3 months)	765 µm	Epidermis+dermis	Dermatomed skin	20% ethanol in 0.05 M phosphate buffer (pH=7.4)	NF	0.1 mg/ml	0.05 M phosphate buffer	12.1 mL	28 h	J = 0.361 µg/cm².h Lag time: 3 to 5 h	NF	N N	Liquid scintillation counting	1.77 cm²	
Gottingen Minipigs (6 months)	765 µm	Epidermis+dermis	Dermatomed skin	20% ethanol in 0.05 M phosphate buffer (pH=7.4)	NF	0.1 mg/ml	0.05 M phosphate buffer	12.1 mL	28 h	J = 0.538 µg/cm².h Lag time: 3 to 5 h	NF	N N	Liquid scintillation counting	1.77 cm²	
Domestic pigs	765 µm	Epidermis+dermis	Dermatomed skin	20% ethanol in 0.05 M phosphate buffer (pH=7.4)	NF	0.1 mg/ml	0.05 M phosphate buffer	12.1 mL	28 h	J = 0.792 µg/cm².h Lag time: 3 to 5 h	NF	N N	Liquid scintillation counting	1.77 cm²	
Human skin (abdominal and breast skin)	408 µm	Epidermis+dermis	Dermatomed skin	20% ethanol in 0.05 M phosphate buffer (pH=7.4)	NF	0.1 mg/ml	0.05 M phosphate buffer	12.1 mL	28 h	J = 0.501 µg/cm².h Lag time: 3 to 5 h	NF	N N	Liquid scintillation counting	1.77 cm²	

Mice skin	-	Full-thickness skin	Surgical materials	PEG 400 (5%, w/v) in saline	5 mL (containing 5 mg TST)	1 mg/mL	PBS/PEG 400 (95/5, V/V)	17.5mL	12 h	J = 0.25 µg/cm^2.h P = 2.49 x 10^{-4} cm/h	NF	N N	HPLC 10µL	1.33 cm²	(Zhang et al., 2017)
Mice skin	-	Full-thickness skin	Surgical materials	Imidazolium ionic liquids and PEG 400 (5%, w/v) in water *Assay were performed with 20 different types of imidazolium ionic liquids	5 mL (containing 5 mg TST)	1 mg/mL	PBS/PEG 400 (95/5, V/V)	17.5mL	12 h	J = 0.42 to 0.9 µg/cm^2.h (range) P= 4.18 to 8.95 x 10^{-4} cm/h	NF	N N	HPLC 10µL	1.33 cm²	
Human skin (abdominal)	400 ± 50 µm	Edpiermis+dermis	Dermatomed	Pure ethanol	10 µL	1.64 µg/cm^2	0.9% NaCl in water + 1% (m/V) BSA + 0.05% (V/V) gentamycin sulfate	NF	24 h	$Q_{permeated}$ = 4.7% of applied dose	NF	N N	Scintillation counting	1.00 cm²	(Hewitt et al., 2020)
Reconstructed skin model (*in vitro*)	-	Epidermis+dermis	Cell cultivation without alpha-linolenic acid and linoleic acid	TST solubilized in Ethanol/water (1/1, V/V)	100 µL (400 µg TST)	4 mg/mL	PBS + 5% bovine serum albumin	5 mL	24 h	J = 78.3 and 52.9 µg/cm^2.h (up to 2 h – two different experimental groups)	NF	N Y (4°C)	UPLC 5 µL	0.63 cm²	(Simard et al., 2019)
Reconstructed skin model (*in vitro*)	-	Epidermis+dermis	Cell cultivation with alpha-linolenic acid (ALA)	TST solubilized in Ethanol/water (1/1, V/V)	100 µL (400 µg TST)	4 mg/mL	PBS + 5% bovine serum albumin	5 mL	24 h	J = 46.8 µg/cm^2.h (up to 2 h)	NF	N Y (4°C)	UPLC 5 µL	0.63 cm²	
Reconstructed skin model (*in vitro*)	-	Epidermis+dermis	Cell cultivation with linolenic acid (ALA)	TST solubilized in Ethanol/water (1/1, V/V)	100 µL (400 µg TST)	4 mg/mL	PBS + 5% bovine serum albumin	5 mL	24 h	J = 48.8 µg/cm^2.h (up to 2 h)	NF	N Y (4°C)	UPLC 5 µL	0.63 cm²	
Silicone membrane	0.05 cm	HT-6240 BISCO® transparent membrane	-	TST solubilized in EtOH:PBS (50: 50)	900 µL	7.07 x 10^3 µg/mL	EtOH: PBS (50: 50)	5 mL	8 h	J = 64 µg/cm^2 P = 9.2 x 10^{-3} cm/h Lag time = 0.22 h	NF	N N	UV spectroscopy (245 nm)	0.64 cm²	(Alberti et al., 2017)
Human skin (abdominal)	1000 µm	Full-thickness skin	Dermatomed skin	TST solubilized in ethanol/water 1/1 (v/v)	25 µL/cm²	4 mg/mL	5% BSA in water	4 mL	24 h	P = 69.3 x10^{-5} cm/h Lag time = 4.4	1.7%	Y (ethanol) N	Liquid scintillation counting	1 cm²	(Guth et al., 2015)

Human skin	400 µm	Epidermis+dermis	Dermatomed skin	80% of the maximal solubility in a 50/50 ethanol/H2O (% V/V)	500 µL	9.71 mg/mL	PBS + 5% (m/V) bovine serum albumin	5 mL	24 h	3.92 x 10^{-4} cm/h	NF	Y (acetonitrile) N	HPLC 25 µL	0.64 cm²	(Veryser et al., 2015)
Human skin	400 µm	Epidermis+dermis	Dermatomed skin	80% of the maximal solubility in a 50/50 ethanol/H2O (% V/V) + Azone (1%, W/V)	500 µL	9.71 mg/mL	PBS + 5% (m/V) bovine serum albumin	5 mL	24 h	25.5 x 10^{-4} cm/h	NF	Y (acetonitrile) N	HPLC 25 µL	0.64 cm²	
Human skin	400 µm	Epidermis+dermis	Dermatomed skin	80% of the maximal solubility in a 50/50 ethanol/H2O (% V/V) + phytoceramides (1%, W/V)	500 µL	9.71 mg/mL	PBS + 5% (m/V) bovine serum albumin	5 mL	24 h	2.05 to 7.02 x 10^{-4} cm/h (range) 10 phytoceramides were tested as chemical absorption enhancers	NF	Y (acetonitrile) N	HPLC 25 µL	0.64 cm²	
Human skin	1) 500 µm 2) 900-1100 µm	Epidermis+dermis	1) Dermatomed skin 2) Full-thickness skin	TST solubilized in ethanol 50% (V/V)	16 µL	4 mg/mL	Saline + 5% (w/v) bovine serum albumin, pH 7.4	0.4 mL	24	1) 1.82 µg/cm².h (lag time = 0.6 h) 2) 0.18 µg/cm².h (lag time = 2.2 h)	1) 136 µg /mL 2) 112 µg/mL	N	Liquid scintillation counting	0.64 cm²	(Wilkinson et al., 2006)

7. ASSAY TOTAL DURATION & SAMPLING INTERVALS

Considering that the TST presents a low diffusion rate through different skin layers, it is rational to consider a long experimental time in permeation studies. Moreover, the more effective barrier effect of the membrane, the longer the time of experiment required to obtain the permeation data for a certain compound. In fact, most of the permeation assays with TST were performed over a time equal or greater than 24 h (Table 2). One the other hand, a time exposure of 12 h or less was enough for permeation assays performed with tissues characterized by greater permeation as mice, rat and amphibian skin. Therefore, the assay total duration depends mainly on the selected membrane model. The distribution of the sampling intervals should be as homogeneous as possible and should not consider too many points. Permeation studies with a sampling time at 12 h and the next at 24 h can be found, however, if any operator error occurs during sampling, all permeation parameters (flux, permeation coefficient and lag time) may be erroneously calculated. Therefore, a homoscedastic distribution of sampling intervals should be designed. Overall, the concentration of TST in receptor fluids was evaluated at time intervals of 0, 1, 2, 4, 8, 12, 16 18, 20, 21 and 24 h in studies with human skin (Baert et al., 2012; Hewitt et al., 2020; Veryser et al., 2015) Another problem for drugs as TST is the inclusion of many sampling intervals. For each replacement of the medium of the receptor phase, the drug is diluted. In these situations, if the quantification method is not sensitive enough, TST may not be detected. This problem occurs particularly when various initial sampling intervals are defined (drug is found in the least amount in the medium in these periods).

8. SKIN INTEGRITY ASSAYS

The evaluation of tissue integrity is strongly recommended for permeation tests involving long incubation periods, such as those considered for the TST. Permeation assay itself can provide indications of the impairment of barrier properties. A change in tissue structure can result in permeability changes (Bennion et al., 2017). If the permeation rate is modified over the course of the assay or an extremely large amount of drug permeates through membrane in specific intervals, this fact may indicate a compromise in the barrier properties and further investigations should be carried out.

OECD, WHO and FDA guidelines recommend the evaluation of skin integrity prior to the permeation study to identify samples that affect the tissue barrier function.

In addition to a visual inspection of the skin, the OECD proposes the measure of transepidermal electrical resistance (TEER), transepidermal water loss (TEWL) and tritiated water. No guidance on how to perform and interpret these integrity tests or then reference values for each assay is available in official guidelines.

While physical methods evaluating TEER and TEWL are useful for the selection of tissues for assays (Bartosova e Bajgar, 2012), histological studies allows to identify the region of tissue most affected (e.g. epidermal desquamation, dermal vacuolization).

TEER represents a simple, rapid, and cost-effective method of cellular barrier integrity. TEER measurements based on impedance spectroscopy have proven to be more reliable and provide more information about the cells when compared to the Ohm's law method (Srinivasan et al., 2015). The measured resistance is dependent on the device, applied frequency, resulting current, ionic strength of the solution, temperature and surface area of the tissue (Guth et al., 2015). Skin samples from different animal models may also affect TEER values. In a study with human, mouse, guinea pig, pig, rat and rabbit skin, TEER values were ≥10, 5, 5, 4, 3 and 0.8 kΩ, respectively (Davies et al., 2004). Kaufmann and Dohmen (2016), for instance, used TEER to evaluate the integrity of fresh excised and frozen amphibian skin. The higher impedance measurements for fresh skin suggested that the freezing step affected the epidermal integrity. In fact, the ice crystal formation in the frozen skin can lead to the disruption of epidermal cells, impairment the tight junctions, and hence impedance decreases. The K_p value of TST through the fresh dorsal skin was lower (1.3×10^{-3} $cm.h^{-1}$) than frozen skin (1.9×10^{-3} $cm.h^{-1}$). On the other hand, a minor transition between intact and damaged skin can be found due to the low impedance of amphibian skin and thus methods such as TEWL would be recommended for analysis of amphibian skin integrity.

TEWL methods measure water vapor flux in the air above the SC, which is an indicator of water diffusion through SC and its barrier properties. A temperature and moisture stabilization time around the probe are required before measurements (Neupane et al., 2020). Another caution refers to the complete removal of moisture on the skin surface generated from rehydration of the frozen skin samples or TEER measurements given that the assay objective is to measure exclusively the water loss from the skin sample (Guth et al., 2015). For human skin, TEWL values ranges from 4

to 10 g/m^2/h depending on the tissue area. When the epidermis is damaged, these values may increase up to 30-times (Boer et al., 2016).

The transepidermal water flux (TWF), which involves the measurement of tritiated water (a radioactive form of water), is another assay able to evaluate the skin barrier function. This test can be carried out before, during or after the permeation assay. When it is performed only before permeation study, eventual tissue damages caused by treatments will not be identified. In this same way, limitations may be found when TWF is performed only at the end of permeation assays. Once the tissue is exposed to infinite amounts of water or hydration for many hours during the permeation and it is often washed after its removal of diffusion cells, which may lead to tissue deterioration, TWF measurements at this stage may reject previously intact tissues (Fabian et al., 2017).

Although these assays detect only changes in the polar transport pathway (Flynn et al., 1974), Guth et al. (2015) considered them during the evaluation of tissue integrity prior to permeation studies with TST. The limit to distinguish damaged and intact tissues was 10 g m^{-2} h^{-1}, 4.5 x 10^{-3} cm h^{-1} and 2 kΩ for TEWL, TWF and TEER, respectively.

The inclusion of chemical markers (e.g., mannitol) whose permeation is already well known have also been considered. In general, two reference compounds presenting different physicochemical characteristics are selected. The results of these tests should be carefully analyzed given that the presence of additional compounds in the donor phase may affect the absorption characteristic of the test compound due to changes in solubility or saturation levels (Guth et al., 2015).

Absorption chemical enhancers are the main agents responsible for compromising the skin barrier properties; however, it is important to highlight that the drug itself may also affect them. For TST, studies have demonstrated that fluctuations in its level modulate barrier function and that hormone replacement with TST can exert a negative consequence for permeability barrier homeostasis (Kao et al., 2001).

9. SAMPLE STORAGE BEFORE QUANTIFICATION

Samples should be quantified immediately after the permeation test has been completed; however, some studies have stored the samples under refrigeration until quantification step (Table 2). It is well known that the storage of drugs presenting low

aqueous solubility under refrigeration can lead to the precipitation and that analytical errors can increase because of a non-soluble drug fraction. On the other hand, storage at room temperature increases the chances of drug degradation by hydrolysis, oxidation and/or enzymatic reactions. Thus, a prior analysis of the drug's stability in different aqueous media and conditions (presence and absence of enzymes, temperature, pH, etc.) is recommended.

10. DRUG QUANTIFICATION STEP

The quantification of steroids from biological matrices (e.g. blood, plasma, urine and skin homogenate) usually involves extraction as the first step and such procedures are carried out by selecting specific solvents. The liquid-liquid extraction may lead to emulsion formation, which would be avoided by a centrifugation step; however, this latter is time-consuming. During the selection of extraction solvent, both polarity of the steroid and its interaction with binding proteins should be considered. Solvents such as acetonitrile and methanol act by providing the disruption of steroid-protein binding through a mechanism of protein denaturation. After solvent addition, the mixture is vortexed, and the protein plug removed by centrifugation (Makin et al., 2010). The protein precipitation efficiency is associated with the solvent type and solvent/biological matrix ratio used. Overall, a ratio 2:1 of solvent (acetonitrile or trichloroacetic acid) to biological matrix is able to precipitate more than 90% of proteins (Polson et al., 2003). In human skin matrices containing TST, Baert et al. (2012) removed proteins by adding a ratio 1:1 of acetonitrile/matrix. No analytical problems were reported in this study.

The absence of proteins in the supernatant may be confirmed by adding a dye capable of binding proteins. When proteins are found, Coomassie blue dye under acidic conditions changes the color from brown to blue (Rodger e Sanders, 2017). The dye binds to basic amino acids by a combination of hydrophobic interactions and heteropolar bonding (Drabik et al., 2016).

In addition to liquid-liquid extraction, solid-phase extraction (SPE) has been often applied to separate the steroids from biological interferences. It is composed by a sorbent such as microparticulate silica coated with octadecasilane, which is packed into syringes or cartridges (Makin et al., 2010). In the case of TST, which has an extremely

low permeation rate and the volumes collected cannot be large, the application of this method is limited. The large number of samples also makes this method expensive.

HPLC has been the most important technique to quantify steroids in different matrices; however, not enough sensitivity could be achieved when a UV detector is used (Makin et al., 2010). Baert et al. (2012), for example, found a quantification limit of 0.17 μg/mL for TST by using this technique. Once the TST has an extremely low initial permeation rate (in the nanogram range) and several authors have considered HPLC as a quantification method, initial time intervals are more susceptible to analytical errors.

If reversed-phase columns are selected, guard column (30-70 mm in length) should be included to avoid the accumulation of non-polar material on the columns or time-consuming clean-up procedures of sample or column. LC-MS-MS (i.e. tandem mass spectrometry) may represent an alternative to overcome this extensive need of clean-up of samples and also is able to provide a lower quantification limit than HPLC-UV (Makin et al., 2010).

Another alternative to improve the sensitivity of the quantification method is to increase the amount of sample injected, however, this must be compatible with the size of column used. In our database for TST (Table 2), most of the authors used an injection volume of 20 μL and a non-processing of the samples before injection (without solvent extraction or SPE). When the injection volume is increased, a greater need for sample clean-up/purification steps should be considered.

11. DRUG RETENTION

Androgen receptors are expressed in sebocytes, hair follicle, dermal papilla and keratinocytes. Hence, TST plays an important role in the sebum production, control hair growth as well as hair loss, epidermal growth and differentiation (Choudhry et al., 1992). The mechanism of action of TST occurs by its local conversion to dihydrotestosterone (DHT) by 5α-reductase (type 1). DHT then binds to the androgen receptor. This means that the increase of TST lead to an increase of DHT concentrations, which is associated with hair loss (alopecia) (Riggs et al., 2002; Shapiro e Price, 1998). The action of this hormone is regulated by cell-type-specific activation or deactivation in human skin. In physiological conditions, fibroblasts present in the

dermis activate mainly TST to DHT. In contrast, keratinocytes present in the epidermis deactivate TST, forming androstenedione (Münster et al., 2003).

The higher concentration of TST in men is associated with a thicker epidermis, and greater amount of collagen and moisture compared to women skin (Markova et al., 2004). On the other hand, the higher levels of TST in male stimulate a large production of sebum, leading to fatty glow and coarser pores of the skin (Baumann, 2002). TST can also perturbs the epidermal barrier homeostasis considering that studies showed a retard in barrier development of skin fetus and slowed barrier recovery in adult skin (Kao et al., 2001). Other negative effects include the inhibition of skin wound healing in males and the TST association with an enhanced inflammatory response (Ashcroft e Mills, 2002). In contrast, a reduction in physiological levels of the hormone has also a negative impact on epidermal skin moisture, elasticity, and thickness (Bernard et al., 2012). In these cases, the local hormone replacement with TST can be advantageous to improve the aspect of skin aging in men.

Transdermal products containing 1% TST are commonly used to reproduce diurnal physiological variations of the hormone in the treatment of male hypogonadism (Mazer, 2000). Once application of TST in skin is focused on the systemic treatment, many *in vitro* studies do not evaluate the presence of the hormone in skin homogenates. Magnusson et al. (2006), for instance, evaluated the distribution of TST in human skin using excised epidermis, dermis, and full-thickness skin. TST retention was higher in the uppermost skin layers (SC > epidermis> dermis), which is attributed to its lipophilicity. In hormone retention assays performed with viable epidermis *versus* full-thickness skin, the dermis layer seemed to restrict TST permeation. The TST retention in viable epidermis from full-thickness skin as a membrane model was 3.36-fold higher than only in epidermis (isolated tissue). Heylings et al. (2018) also reported a lower affinity of the TST for dermis. The authors found 0.41 and 0.32% of dose applied of TST (1.0 mg/mL) in epidermis and dermis, respectively.

TST has a great affinity for the lipophilic layers of the skin, contributing for its accumulation in the tissue. This could generate important adverse effects from a topical administration. However, studies have not thoroughly investigated the possible toxic effects of this hormone in skin.

12. FINAL CONSIDERATIONS

The interplay/tradeoff solubility-permeation should not ignored and researchers need to achieve an optimal solubility-permeation balance to maximize the permeation of poorly soluble drugs such as TST. The inclusion of ethanol in aqueous medium increased the solubility or free fraction of TST in the solution, which solves problems of solubility in receptor fluids and maintains sink conditions; however, a reduction in its permeation rate was found when compared to aqueous solutions without solubilizing agents. In this same way, an optimal supersaturation degree should be found to maximize permeation and avoid drug precipitation phenomena. Infinite doses have been frequently used in commercial preparations of hormone due to its low cutaneous absorption; however, an increase in permeation is not always achieved with this approach as reported here.

The type of tissue selected as the membrane model as well as its preparation has also been shown to strongly affect the TST permeation rate. When full-thickness skin is considered, the dermis acts as a barrier that restricts the diffusion of TST, significantly reducing the partitioning into the receptor chamber. Therefore, the amount of absorbed TST is underestimated. The drug retention in epidermis is also increased in an unrealistic way. This fact represents the main reason for prioritizing dermatomized skin. Substantial differences in the rate of transport of the hormone were found depending on the animal model and between biological and artificial membranes/tissues. Despite its widespread use, mouse/mice skin is much more permeable than human skin and should not be prioritized in initial studies (when the permeation of a certain molecule is not yet known). Human skin and pig skin are more suitable in these situations. Mouse/mice skin can be useful, for example, for comparing the performance of different formulations and *in vivo* distribution studies. Likewise, the TST permeation results obtained with artificial membranes are quite different those with human skin.

Although various cutaneous permeation studies with TST were presented in this review, few studies evaluating its retention in the dermis and epidermis are reported. As already mentioned, the skin presents specific receptors for TST, which makes this type of study even more important. This fact could be justified by the complexity of separating the epidermis from the dermis. As already mentioned, the currently available methods that use heating or chemical treatment can affect tissue integrity. For this

reason, manual tissue separation has been proposed, but poorly reproducible results are usually found.

Although traditional methods in the pharmaceutical field have been considered for the quantification of TST in biological matrices, in-depth studies of sample preparation are still scarce. Improvements in terms of the method sensitivity could be achieved with an adequate processing of the samples, reducing the experimental time and the data generated would also be more reliable.

In summary, this review contributes to define more properly the experimental variables of cutaneous permeation assays with poorly soluble molecules such as the TST, creating new discussions for future revisions of official guidelines.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

ACKNOWLEDGEMENTS

We gratefully acknowledge the Brazilian governmental agencies CNPq/MCTI (Universal 01/2016 - Grant number: 408229/2016-0) and CAPES for financial support and student scholarships, respectively.

REFERENCES

Abd, E., Yousef, S.A., Pastore, M.N., Telaprolu, K., Mohammed, Y.H., Namjoshi, S., Grice, J.E., Roberts, M.S., 2016. Skin models for the testing of transdermal drugs. Clin. Pharmacol. Adv. Appl. 8, 163–176. https://doi.org/10.2147/CPAA.S64788

Ainbinder, D., Touitou, E., 2005. Testosterone ethosomes for enhanced transdermal delivery. Drug Deliv. 12, 297–303.

Alberti, M., Dancik, Y., Sriram, G., Wu, B., Teo, Y.L., Feng, Z., Bigliardi-Qi, M., Wu, R.G., Wang, Z.P., Bigliardi, P.L., 2017. Multi-chamber microfluidic platform for high-precision skin permeation testing. Lab Chip 17, 1625–1634. https://doi.org/10.1039/c6lc01574c

Araya, H., Tomita, M., Hayashi, M., 2005. The novel formulation design of O/W microemulsion for improving the gastrointestinal absorption of poorly water soluble compounds. Int. J. Pharm. 305, 61–74. https://doi.org/10.1016/j.ijpharm.2005.08.022

Ashcroft, G.S., Mills, S.J., 2002. Androgen receptor–mediated inhibition of cutaneous wound healing. J. Clin. Invest. 110, 615–624. https://doi.org/10.1172/JCI15704

Azarmi, S., Roa, W., Löbenberg, R., 2007. Current perspectives in dissolution testing of conventional and novel dosage forms. Int. J. Pharm. 328, 12–21. https://doi.org/10.1016/j.ijpharm.2006.10.001

Baert, B., Roche, N., Burvenich, C., De Spiegeleer, B., 2012. Increase of the transdermal penetration of testosterone by miconazole nitrate. Arch. Pharm. Res. 35, 2163–2170. https://doi.org/10.1007/s12272-012-1214-4

Barbero, A.M., Frasch, H.F., 2016. Effect of Frozen Human Epidermis Storage Duration and Cryoprotectant on Barrier Function Using Two Model Compounds. Skin Pharmacol. Physiol. 29, 31–40. https://doi.org/10.1159/000441038

Bartosova, L., Bajgar, J., 2012. Transdermal Drug Delivery In Vitro Using Diffusion Cells. Curr. Med. Chem. 19, 4671–4677. https://doi.org/10.2174/092986712803306358

Baumann, G., 2002. Growth hormone binding protein. The soluble growth hormone receptor. Minerva Endocrinol. 27, 265–76.

Bennion, B.J., Be, N.A., McNerney, M.W., Lao, V., Carlson, E.M., Valdez, C.A., Malfatti, M.A., Enright, H.A., Nguyen, T.H., Lightstone, F.C., Carpenter, T.S., 2017. Predicting a Drug's Membrane Permeability: A Computational Model Validated With in Vitro Permeability Assay Data. J. Phys. Chem. B 121, 5228–5237. https://doi.org/10.1021/acs.jpcb.7b02914

Bernard, P., Scior, T., Do, Q.T., 2012. Modulating testosterone pathway: a new strategy to tackle male skin aging? Clin. Interv. Aging 351. https://doi.org/10.2147/CIA.S34034

Binks, B.P., Fletcher, P.D.I., Johnson, A.J., Elliott, R.P., 2012. Membrane Permeation

of Testosterone from Either Solutions, Particle Dispersions, or Particle-Stabilized Emulsions. Langmuir 28, 2510–2522. https://doi.org/10.1021/la204755m

Blythe, L. Lou, Gannon, J.R., Craig, A.M., 1994. Care of the racing greyhound. American Greyhound Council.

Boer, M., Duchnik, E., Maleszka, R., Marchlewicz, M., 2016. Structural and biophysical characteristics of human skin in maintaining proper epidermal barrier function. Adv. Dermatology Allergol. 1, 1–5. https://doi.org/10.5114/pdia.2015.48037

Bonina, F.P., Carelli, V., Di Colo, G., Montenegro, L., Nannipieri, E., 1993. Vehicle effects on in vitro skin permeation of and stratum corneum affinity for model drugs caffeine and testosterone. Int. J. Pharm. 100, 41–47. https://doi.org/10.1016/0378-5173(93)90073-O

Bronaugh, R.L., Stewart, R.F., 1985. Methods for in vitro percutaneous absorption studies IV: The flow-through diffusion cell. J. Pharm. Sci. 74, 64–67.

Carrer, V., Alonso, C., Pont, M., Zanuy, M., Córdoba, M., Espinosa, S., Barba, C., Oliver, M.A., Martí, M., Coderch, L., 2020. Effect of propylene glycol on the skin penetration of drugs. Arch. Dermatol. Res. 312, 337–352.

Caussin, J., Gooris, G.S., Janssens, M., Bouwstra, J.A., 2008. Lipid organization in human and porcine stratum corneum differs widely, while lipid mixtures with porcine ceramides model human stratum corneum lipid organization very closely. Biochim. Biophys. Acta - Biomembr. 1778, 1472–1482. https://doi.org/10.1016/j.bbamem.2008.03.003

Ceschel, G., Bergamante, V., Maffei, P., Borgia, S.L., Calabrese, V., Biserni, S., Ronchi, C., 2005. Solubility and Transdermal Permeation Properties of a Dehydroepiandrosterone Cyclodextrin Complex from Hydrophilic and Lipophilic Vehicles. Drug Deliv. 12, 275–280. https://doi.org/10.1080/10717540500176563

Choudhry, R., Hodgins, M.B., Van der Kwast, T.H., Brinkmann, A.O., Boersma, W.J.A., 1992. Localization of androgen receptors in human skin by immunohistochemistry: implications for the hormonal regulation of hair growth, sebaceous glands and sweat glands. J. Endocrinol. 133, 467-NP. https://doi.org/10.1677/joe.0.1330467

Cilurzo, F., Musazzi, U.M., Franzé, S., Fedele, G., Minghetti, P., 2018. Design of in vitro skin permeation studies according to the EMA guideline on quality of transdermal patches. Eur. J. Pharm. Sci. 125, 86–92. https://doi.org/10.1016/j.ejps.2018.09.014

Clowes, H.M., Scott, R.C., Heylings, J.R., 1994. Skin absorption: flow-through or static diffusion cells. Toxicol. Vitr. 8, 827–830.

Couto, A., Fernandes, R., Cordeiro, M.N.S., Reis, S.S., Ribeiro, R.T., Pessoa, A.M., 2014. Dermic diffusion and stratum corneum: A state of the art review of mathematical models. J. Control. Release 177, 74–83. https://doi.org/10.1016/j.jconrel.2013.12.005

Davies, D.J., Ward, R.J., Heylings, J.R., 2004. Multi-species assessment of electrical resistance as a skin integrity marker for in vitro percutaneous absorption studies.

Toxicol. Vitr. 18, 351–358. https://doi.org/10.1016/j.tiv.2003.10.004

Drabik, A., Bodzoń-Kułakowska, A., Silberring, J., 2016. 7 - Gel Electrophoresis, in: Ciborowski, P., Silberring, J.B.T.-P.P. and A.C. (Second E. (Orgs.), . Elsevier, Boston, p. 115–143. https://doi.org/https://doi.org/10.1016/B978-0-444-63688-1.00007-0

EMA, 2012. https://www.ema.europa.eu/en/documents/scientific-guideline/draft-g%0Auideline-quality-transdermal-patches_en.pdf

Fabian, E., Oesch, F., Ott, K., Landsiedel, R., van Ravenzwaay, B., 2017. A protocol to determine dermal absorption of xenobiotica through human skin in vitro. Arch. Toxicol. 91, 1497–1511. https://doi.org/10.1007/s00204-016-1782-z

Fahmy, F.S., Navsaria, H.A., Frame, J.D., Jones, C.R., Leigh, I.M., 1993. Skin graft storage and keratinocyte viability. Br. J. Plast. Surg. 46, 292–295. https://doi.org/10.1016/0007-1226(93)90005-V

Fang, J.-Y., Hwang, T.-L., Fang, C.-L., Chiu, H.-C., 2003. In vitro and in vivo evaluations of the efficacy and safety of skin permeation enhancers using flurbiprofen as a model drug. Int. J. Pharm. 255, 153–166. https://doi.org/10.1016/S0378-5173(03)00086-3

Finnin, B., Walters, K.A., Franz, T.J., 2012. In Vitro Skin Permeation Methodology, in: Benson, A.E., H., Watkinson, A.C. (Orgs.), Transdermal and Topical Drug Delivery: Principles and Practice. John Wiley & Sons, Inc, p. 85–108.

Flynn, G.L., Yalkowsky, S.H., Roseman, T.J., 1974. Mass transport Phenomena and Models: Theoretical Concepts. J. Pharm. Sci. 63, 479–510. https://doi.org/10.1002/jps.2600630403

Guth, K., Schäfer-Korting, M., Fabian, E., Landsiedel, R., van Ravenzwaay, B., 2015. Suitability of skin integrity tests for dermal absorption studies in vitro. Toxicol. Vitr. 29, 113–123. https://doi.org/10.1016/j.tiv.2014.09.007

Haq, A., Michniak-Kohn, B., 2018. Effects of solvents and penetration enhancers on transdermal delivery of thymoquinone: Permeability and skin deposition study. Drug Deliv. 25, 1943–1949.

Hewitt, N.J., Grégoire, S., Cubberley, R., Duplan, H., Eilstein, J., Ellison, C., Lester, C., Fabian, E., Fernandez, J., Géniès, C., Jacques-Jamin, C., Klaric, M., Rothe, H., Sorrell, I., Lange, D., Schepky, A., 2020. Measurement of the penetration of 56 cosmetic relevant chemicals into and through human skin using a standardized protocol. J. Appl. Toxicol. 40, 403–415. https://doi.org/10.1002/jat.3913

Heylings, J R, Davies, D.J., Burton, R., 2018. Dermal absorption of testosterone in human and pig skin in vitro. Toxicol. Vitr. 48, 71–77. https://doi.org/10.1016/j.tiv.2017.12.014

Horita, D., Todo, H., Sugibayashi, K., 2012. Effect of Ethanol Pretreatment on Skin Permeation of Drugs. Biol. Pharm. Bull. 35, 1343–1348. https://doi.org/10.1248/bpb.b12-00293

Houghton, E., Maynard, S., 2010. Some aspects of doping and medication control in equine sports. Doping Sport. Biochem. Princ. Eff. Anal. 369–409.

Hueber, F., Schaefer, H., Wepierre, J., 1994. Role of transepidermal and transfollicular routes in percutaneous absorption of steroids: In vitro studies on human skin. Skin Pharmacol. Physiol. 7, 237–244. https://doi.org/10.1159/000211300

Hueber, F., Wepierre, J., Schaefer, H., 1992. Role of transepidermal and transfollicular routes in percutaneous absorption of hydrocortisone and testosterone: in vivo study in the hairless rat. Skin Pharmacol. Physiol. 5, 99–107.

Imai, M., Hashizaki, K., Yanagi, A., Taguchi, H., Motohashi, Y.S.S., Fujii, M., 2016. Skin permeation of testosterone from viscoelastic lecithin reverse wormlike micellar solution. Biol. Pharm. Bull. 39, 532–539. https://doi.org/10.1248/bpb.b15-00750

Ishii, H., Todo, H., Sugibayashi, K., 2010. Effect of Thermodynamic Activity on Skin Permeation and Skin Concentration of Triamcinolone Acetonide. Chem. Pharm. Bull. (Tokyo). 58, 556–561. https://doi.org/10.1248/cpb.58.556

Jacobi, U., Kaiser, M., Toll, R., Mangelsdorf, S., Audring, H., Otberg, N., Sterry, W., Lademann, J., 2007. Porcine ear skin: an in vitro model for human skin. Ski. Res. Technol. 13, 19–24. https://doi.org/10.1111/j.1600-0846.2006.00179.x

Kaminetsky, J., Wynia, B., 2015. Current and emerging testosterone therapies for male hypogonadism. Res. Reports Endocr. Disord. 59. https://doi.org/10.2147/RRED.S46193

Kao, J.S., Garg, A., Mao-Qiang, M., Crumrine, D., Ghadially, R., Feingold, K.R., Elias, P.M., 2001. Testosterone Perturbs Epidermal Permeability Barrier Homeostasis. J. Invest. Dermatol. 116, 443–451. https://doi.org/10.1046/j.1523-1747.2001.01281.x

Kaplun-Frischoff, Y., Touitou, E., 1997. Testosterone skin permeation enhancement by menthol through formation of eutectic with drug and interaction with skin lipids. J. Pharm. Sci. 86, 1394–1399. https://doi.org/10.1021/js9701465

Kaufmann, K., Dohmen, P., 2016. Adaption of a dermal in vitro method to investigate the uptake of chemicals across amphibian skin. Environ. Sci. Eur. 28. https://doi.org/10.1186/s12302-016-0080-y

KIM, M.-K., LEE, C.-H., KIM, D.-D., 2000. Skin Permeation of Testosterone and its Ester Derivatives in Rats. J. Pharm. Pharmacol. 52, 369–375. https://doi.org/10.1211/0022357001774101

Kim, M.K., Zhao, H., Lee, C.H., Kim, D.D., 2001. Formulation of a reservoir-type testosterone transdermal delivery system. Int. J. Pharm. 219, 51–59. https://doi.org/10.1016/S0378-5173(01)00631-7

Knorr, F., Lademann, J., Patzelt, A., Sterry, W., Blume-Peytavi, U., Vogt, A., 2009. Follicular transport route–research progress and future perspectives. Eur. J. Pharm. Biopharm. 71, 173–180.

Kretsos, K., Miller, M.A., Zamora-Estrada, G., Kasting, G.B., 2008. Partitioning, diffusivity and clearance of skin permeants in mammalian dermis. Int. J. Pharm. 346, 64–79. https://doi.org/10.1016/j.ijpharm.2007.06.020

Krugluger, W., Rohrbacher, W., Laciak, K., Moser, K., Moser, C., Hugeneck, J., 2005. Reorganization of hair follicles in human skin organ culture induced by cultured

human follicle-derived cells. Exp. Dermatol. 14, 580–585.

Land, L.M., Li, P., Bummer, P.M., 2005. The Influence of Water Content of Triglyceride Oils on the Solubility of Steroids. Pharm. Res. 22, 784–788. https://doi.org/10.1007/s11095-005-2595-6

Lauterbach, A., Müller-Goymann, C.C., 2015. Applications and limitations of lipid nanoparticles in dermal and transdermal drug delivery via the follicular route. Eur. J. Pharm. Biopharm. 97, 152–163.

Leichtnam, M.-L., Rolland, H., Wüthrich, P., Guy, R.H., 2006. Enhancement of transdermal testosterone delivery by supersaturation. J. Pharm. Sci. 95, 2373–2379. https://doi.org/10.1002/jps.20669

Leichtnam, M.L., Rolland, H., Wüthrich, P., Guy, R.H., 2006. Identification of penetration enhancers for testosterone transdermal delivery from spray formulations. J. Control. Release 113, 57–62. https://doi.org/10.1016/j.jconrel.2006.03.008

Lillywhite, H.B., 2006. Water relations of tetrapod integument. J. Exp. Biol. 209, 202–226. https://doi.org/10.1242/jeb.02007

Llewelyn, V.K., Berger, L., Glass, B.D., 2019. Permeability of frog skin to chemicals: effect of penetration enhancers. Heliyon 5, e02127. https://doi.org/10.1016/j.heliyon.2019.e02127

Lu, W., Luo, H., Wu, Y., Zhu, Z., Wang, H., 2013. Preparation and characterization of a metered dose transdermal spray for testosterone. Acta Pharm. Sin. B 3, 392–399. https://doi.org/10.1016/j.apsb.2013.10.003

Magnusson, B.M., Cross, S.E., Winckle, G., Roberts, M.S., 2006. Percutaneous Absorption of Steroids: Determination of in vitro Permeability and Tissue Reservoir Characteristics in Human Skin Layers. Skin Pharmacol. Physiol. 19, 336–342. https://doi.org/10.1159/000095254

Makin, H.L.J., Honour, J.W., Shackleton, C.H.L., Griffiths, W.J., 2010. General Methods for the Extraction, Purification, and Measurement of Steroids by Chromatography and Mass Spectrometry BT - Steroid Analysis, in: Makin, H.L.J., Gower, D.B. (Orgs.), . Springer Netherlands, Dordrecht, p. 163–282. https://doi.org/10.1023/b135931_3

Markova, M.S., Zeskand, J., McEntee, B., Rothstein, J., Jimenez, S.A., Siracusa, L.D., 2004. A Role for the Androgen Receptor in Collagen Content of the Skin. J. Invest. Dermatol. 123, 1052–1056. https://doi.org/10.1111/j.0022-202X.2004.23494.x

Mazer, N.A., 2000. New clinical applications of transdermal testosterone delivery in men and women. J. Control. Release 65, 303–315. https://doi.org/10.1016/S0168-3659(99)00252-7

McFarlane, R.M., DeYoung, G., Henry, R.A., McFarlane, R.M., 1965. The design of a pedicle flap in the rat to study necrosis and its prevention. Plast. Reconstr. Surg. 35, 177–182. https://doi.org/10.1097/00006534-196502000-00007

Michel, M., L'Heureux, N., Pouliot, R., Xu, W., Auger, F.A., Germain, L., 1999. Characterization of a new tissue-engineered human skin equivalent with hair. Vitr.

Cell. Dev. Biol. 35, 318.

Mills, P.C., 2007. Vehicle effects on the In vitro penetration of testosterone through equine skin. Vet. Res. Commun. 31, 227–233. https://doi.org/10.1007/s11259-006-3446-6

Mills, P.C., Magnusson, B.M., Cross, S.E., 2006. The effects of vehicle and region of application on in vitro penetration of testosterone through canine skin. Vet. J. 171, 276–280. https://doi.org/10.1016/j.tvjl.2004.11.013

Moser, K., Kriwet, K., Naik, A., Kalia, Y.N., Guy, R.H., 2001. Passive skin penetration enhancement and its quantification in vitro. Eur. J. Pharm. Biopharm. 52, 103–112. https://doi.org/10.1016/S0939-6411(01)00166-7

Münster, U., Hammer, S., Blume-Peytavi, U., Schäfer-Korting, M., 2003. Testosterone metabolism in human skin cells in vitro and its interaction with estradiol and dutasteride. Skin Pharmacol. Appl. Skin Physiol. 16, 356–366. https://doi.org/10.1159/000072930

Netzlaff, F., Schaefer, U.F., Lehr, C.-M., Meiers, P., Stahl, J., Kietzmann, M., Niedorf, F., 2006. Comparison of bovine udder skin with human and porcine skin in percutaneous permeation experiments. ATLA Altern. to Lab. Anim. 34, 499–513.

Neupane, R., Boddu, S.H.S., Renukuntla, J., Babu, R.J., Tiwari, A.K., 2020. Alternatives to Biological Skin in Permeation Studies: Current Trends and Possibilities. Pharmaceutics 12, 152. https://doi.org/10.3390/pharmaceutics12020152

Ng, S.-F., Rouse, J.J., Sanderson, F.D., Meidan, V., Eccleston, G.M., 2010. Validation of a Static Franz Diffusion Cell System for In Vitro Permeation Studies. AAPS PharmSciTech 11, 1432–1441. https://doi.org/10.1208/s12249-010-9522-9

Nicolazzo, J.A., Morgan, T.M., Reed, B.L., Finnin, B.C., 2005. Synergistic enhancement of testosterone transdermal delivery. J. Control. Release 103, 577–585. https://doi.org/10.1016/j.jconrel.2004.12.007

Norman, A.W., Litwack, G., 1997. Hormones. Academic Press.

OECD, 2004. Guidance Document for the Conduct of Skin Absorption Studies. OECD Series on Testing and Assessment. https://doi.org/10.1787/9789264078796-en

Pelletier, G., Ren, L., 2004. Localization of sex steroid receptors in human skin. Histol. Histopathol. 19, 629–636. https://doi.org/10.14670/HH-19.629

Plumb, D.C., 2002. Meclizine. Veterinary Drug Book, ed 4. Ames.

Polson, C., Sarkar, P., Incledon, B., Raguvaran, V., Grant, R., 2003. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry. J. Chromatogr. B 785, 263–275. https://doi.org/10.1016/S1570-0232(02)00914-5

Qvist, M.H., Hoeck, U., Kreilgaard, B., Madsen, F., Frokjaer, S., 2000. Evaluation of Gottingen minipig skin for transdermal in vitro permeation studies. Eur. J. Pharm. Sci. 11, 59–68. https://doi.org/10.1016/S0928-0987(00)00091-9

Riggs, B.L., Khosla, S., Melton, L.J., 2002. Sex Steroids and the Construction and

Conservation of the Adult Skeleton. Endocr. Rev. 23, 279–302. https://doi.org/10.1210/edrv.23.3.0465

Rodger, A., Sanders, K., 2017. UV-Visible Absorption Spectroscopy, Biomacromolecular Applications, in: Encyclopedia of Spectroscopy and Spectrometry. Elsevier, p. 495–502. https://doi.org/10.1016/B978-0-12-803224-4.00108-4

SCCS, 2010. Basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients. Eur. Comm 1–14.

Schlupp, P., Weber, M., Schmidts, T., Geiger, K., Runkel, F., 2014. Development and validation of an alternative disturbed skin model by mechanical abrasion to study drug penetration. Results Pharma Sci. 4, 26–33. https://doi.org/10.1016/j.rinphs.2014.09.002

Schreiber, S., Mahmoud, A., Vuia, A., Rübbelke, M.K., Schmidt, E., Schaller, M., Kandárová, H., Haberland, A., Schäfer, U.F., Bock, U., Korting, H.C., Liebsch, M., Schäfer-Korting, M., 2005. Reconstructed epidermis versus human and animal skin in skin absorption studies. Toxicol. Vitr. 19, 813–822. https://doi.org/10.1016/j.tiv.2005.04.004

Schwarb, F.P., Imanidis, G., Smith, E.W., Haigh, J., Surber, C., 1999. Effect of Concentration and Degree of Saturation of Topical Fluocinonide Formulations on In Vitro Membrane Transport and In Vivo Availability on Human Skin. Pharm. Res. 16, 909–15. https://doi.org/10.1023/A:1018890422825

Schwarz, D.H., Engelke, A., Wenz, G., 2017. Solubilizing steroidal drugs by β-cyclodextrin derivatives. Int. J. Pharm. 531, 559–567. https://doi.org/10.1016/j.ijpharm.2017.07.046

Shapiro, J., Price, V.H., 1998. Hair regrowth. Therapeutic agents. Dermatol. Clin. 16, 341–356. https://doi.org/10.1016/S0733-8635(05)70017-6

Simard, M., Julien, P., Fradette, J., Pouliot, R., 2019. Modulation of the Lipid Profile of Reconstructed Skin Substitutes after Essential Fatty Acid Supplementation Affects Testosterone Permeability. Cells 8. https://doi.org/10.3390/cells8101142

Snow, D.H., 1993. Anabolic Steroids. Vet. Clin. North Am. Equine Pract. 9, 563–576. https://doi.org/10.1016/S0749-0739(17)30386-3

Srinivasan, B., Kolli, A.R., Esch, M.B., Abaci, H.E., Shuler, M.L., Hickman, J.J., 2015. TEER Measurement Techniques for In Vitro Barrier Model Systems. J. Lab. Autom. 20, 107–126. https://doi.org/10.1177/2211068214561025

Swarbrick, J., Lee, G., Brom, J., 1982. Drug Permeation through Human Skin: I. Effects of Storage Conditions of Skin. J. Invest. Dermatol. 78, 63–66. https://doi.org/10.1111/1523-1747.ep12497937

Thomas, N.S., Panchagnula, R., 2003. Transdermal delivery of zidovudine: effect of vehicles on permeation across rat skin and their mechanism of action. Eur. J. Pharm. Sci. 18, 71–79. https://doi.org/10.1016/S0928-0987(02)00242-7

van de Sandt, J.J.M., van Burgsteden, J.A., Cage, S., Carmichael, P.L., Dick, I., Kenyon, S., Korinth, G., Larese, F., Limasset, J.C., Maas, W.J.M., Montomoli, L.,

Nielsen, J.B., Payan, J.-P., Robinson, E., Sartorelli, P., Schaller, K.H., Wilkinson, S.C., Williams, F.M., 2004. In vitro predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study. Regul. Toxicol. Pharmacol. 39, 271–281. https://doi.org/10.1016/j.yrtph.2004.02.004

Veryser, L., Boonen, J., Taevernier, L., Guillaume, J., Risseeuw, M., Shah, S.N.H., Roche, N., Van Calenbergh, S., De Spiegeleer, B., 2015. The influence of the Acyl chain on the transdermal penetration-enhancing effect of synthetic phytoceramides. Skin Pharmacol. Physiol. 28, 124–136. https://doi.org/10.1159/000365730

Wei, J.C.J., Edwards, G.A., Martin, D.J., Huang, H., Crichton, M.L., Kendall, M.A.F., 2017. Allometric scaling of skin thickness, elasticity, viscoelasticity to mass for micro-medical device translation: from mice, rats, rabbits, pigs to humans. Sci. Rep. 7, 15885. https://doi.org/10.1038/s41598-017-15830-7

WHO, 2006. Dermal absorption. World Health Organization.

Wilkinson, S.C., Maas, W.J.M., Nielsen, J.B., Greaves, L.C., van de Sandt, J.J.M., Williams, F.M., 2006. Interactions of skin thickness and physicochemical properties of test compounds in percutaneous penetration studies. Int. Arch. Occup. Environ. Health 79, 405–413. https://doi.org/10.1007/s00420-005-0056-5

Williams, A.C., Barry, B.W., 2012. Penetration enhancers. Adv. Drug Deliv. Rev. 64, 128–137. https://doi.org/10.1016/j.addr.2012.09.032

Zhang, D., Wang, H.-J., Cui, X.-M., Wang, C.-X., 2017. Evaluations of imidazolium ionic liquids as novel skin permeation enhancers for drug transdermal delivery. Pharm. Dev. Technol. 22, 511–520. https://doi.org/10.3109/10837450.2015.1131718

Zhou, L., Yang, L., Tilton, S., Wang, J., 2007. Development of a high throughput equilibrium solubility assay using miniaturized shake-flask method in early drug discovery. J. Pharm. Sci. 96, 3052–3071. https://doi.org/10.1002/jps.20913

Zsikó, S., Csányi, E., Kovács, A., Budai-Szűcs, M., Gácsi, A., Berkó, S., 2019. Methods to evaluate skin penetration in vitro. Sci. Pharm. 87, 19.

4. CAPÍTULO III – EFEITO DOS TERPENOS COMO PROMOTORES DE ABSORÇÃO PARA A TESTOSTERONA

Este capítulo trata da avaliação da permeação percutânea da testosterona em que diferentes terpenos foram selecionados como promotores químicos de absorção. Análises espectroscópicas (FTIR) e térmicas (DSC) foram consideradas para uma investigação de interações específicas entre estes terpenos e componentes da pele (domínios lipídicos e estruturas proteicas) a fim de entender como as propriedades de barreira do estrato córneo são modificadas. O produto deste capítulo é um artigo científico, que está sob avaliação na revista *Journal of Drug Delivery Science and Technology*.

4.1. ARTIGO CIENTÍFICO II

ENHANCING THE PERMEATION OF TESTOSTERONE THROUGH HUMAN SKIN WITH NATURAL TERPENES

Nicole Esposto Biondo[1]; Débora Fretes Argenta[1]; Gabriela Schneider Rauber[1]; Luciano Vitali[2]; Gustavo Amadeu Micke[2]; Thiago Caon[1#]

[1]Postgraduate Program in Pharmacy/[2]Postgraduate Program in Chemistry, Federal University of Santa Catarina, Campus Reitor João David Ferreira Lima, s/n - Trindade, Florianópolis, SC 88040-900, Brazil.

[#]Corresponding author: thiagocaon@gmail.com; caon.thiago@ufsc.br

ABSTRACT

Chemical absorption enhancers (CAEs) may be demanded in formulations with drugs having low diffusion rate or high retention in skin. Although several CAEs are available for commercial use, incompatibility or local irritation issues are common in daily applied formulations. Terpenes has been shown to be promising candidates in this context. In addition to high efficacy as CAE, they are classified as safe by FDA and can provide secondary biological properties (e.g., antioxidant, anti-inflammatory and antimicrobial action). Therefore, the effects of three different terpenes on the cutaneous permeation of testosterone (TST) was investigated by the Franz-type diffusion model. An enhancement ratio of 7.61, 5.10 and 2.52 was obtained after treatment with carvacrol, α-bisabolol and menthol, respectively. Carvacrol provided higher permeation flux and cutaneous retention of TST than other terpenes. Mechanistic studies were performed to evaluate the effect of CAEs on biophysical properties of the stratum corneum. Spectroscopic and thermal analyses showed structural changes in the tissue after all treatments and suggested that carvacrol causes a greater disorder in lipid domains. As TST is preferentially transported through the skin by passive diffusion due to its lipophilic nature, this terpene would be more promising as CAE. This terpene could also play a synergistic effect with TST in hypogonadism, but further studies are needed to prove the advantages of this association.

Keywords: terpenes; skin permeation; testosterone; human skin.

1. INTRODUCTION

The transdermal route has been widely used in recent years due to numerous advantages over the oral once it avoids the first-pass metabolism, protects drugs from the gastrointestinal tract, allows a sustained release of drugs and provides high patient compliance to treatment [1]. This alternative administration route is even more attractive for the testosterone (TST) once it is extensively metabolized after an oral administration. In this situation, large oral doses are required to achieve normal plasma levels, increasing the chances of side effects such as liver toxicity and lowering of HDL cholesterol levels [2].

Numerous derivatives of TST have also been synthesized aiming to extend its *in vivo* biological activity following an oral application. Although these agents are characterized by a high efficacy, behavioral changes (e.g., aggression), hepatotoxicity, modification of blood lipid levels and coagulation factors and some degree of masculinization in women and children are often observed [3].

In this context, the transdermal route has been shown to be a promising alternative to overcome these limitations, which explain the large number of transdermal preparations of TST available on the market in recent years. Transdermal patches and gels represent the most common dosage forms [4]. Given that the TST presents a high value of log P, this compound binds so strongly to the tissues, particularly in the more superficial layers (*stratum corneum* and viable epidermis), resulting in a reservoir effect and low transport rate to the dermis [5]. Moreover, the skin has receptors for this hormone due to the physiological action it plays in that region. The TST interacts directly with androgen receptors, which are localized in most epidermis keratinocytes [6].

In this context, the inclusion of chemical absorption enhancers (CAEs) in transdermal formulation can be considered as an approach to increase the delivery of TST in plasma. Alcohols have been often included in gel formulations to play a role as a CAE and solubilizing agent, but its highly dehydrating nature causes a quick dryness of the epidermis. Consequently, the permeation of TST through the skin may be hampered. In addition, local irritation may be observed [7]. Indeed, in a study with different

concentrations of ethanol in water, lower transport rate of TST through rat skin was observed for increasing amounts of ethanol [8].

Natural terpenes, in turn, would avoid these events and have been pointed out as promising CAE candidates due to their low toxicity compared to other synthetic representatives [9]. As dosage forms usually contain high doses of TST, which also can irritate the skin, terpenes with anti-inflammatory or anti-irritant activity would minimize or even eliminate this problem. In addition, these compounds may play a synergistic action with TST in different organic tissues or provide additional properties in formulations (e.g., antimicrobial, antioxidant and solubilizing action). Therefore, the role of three terpenes (carvacrol, α-bisabolol, and menthol; Figure 1) on the permeation rate of TST through human skin was studied. Spectroscopic and thermal studies were also performed to understand the terpene/skin interactions.

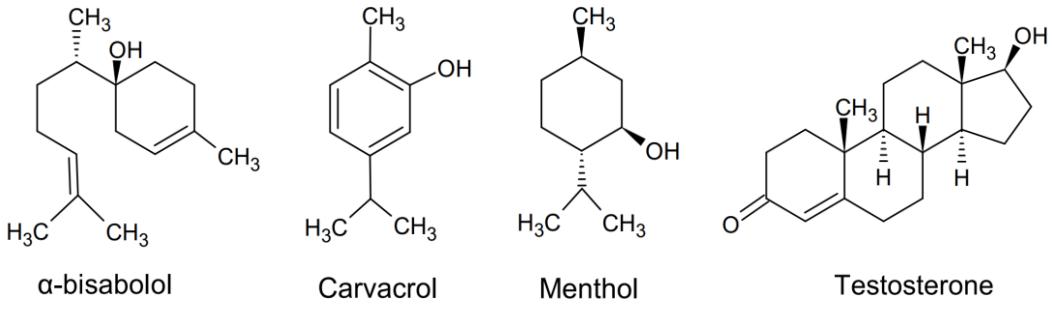


Figure 1. Chemical structures of terpenes and testosterone.

2. MATERIAL AND METHODS

2.1 Materials

TST base (98% purity) and propylene glycol (PG) were obtained from Infinity® Pharma (Campinas, SP, Brazil) and CRQ (Diadema, SP, Brazil), respectively. Carvacrol (98%), α-bisabolol (93%) and menthol (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline was prepared with 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 136.9 mM NaCl, 2.7 mM KCl and adjusted to pH 7.4 with 1 M HCl. Krebs-Ringer bicarbonate buffer was prepared with 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2.2H_2O$, 1.0 mM $MgCl_2.6H_2O$, 5.5 mM glucose and adjusted to pH 7.4 with 1 M HCl. Methanol and acetonitrile were

of HPLC grade. All other chemicals and reagents were of analytical grade. Ultrapure water was obtained from a Milli-Q® purification system (Millipore, Milford, MA, USA).

2.2 Methods

2.2.1 Ex vivo permeation studies

2.2.1.1 Preparation of human skin samples

Human skin was obtained from abdominal surgery performed at a local hospital. The study was approved by the University Research Ethics Committee (CAE/protocol number: 87349418.7.0000.0121). Full-thickness skin was stored in cold Krebs buffer immediately after the surgery. The skin samples were prepared by removing the adipose tissue, placed on aluminum foil sheets and stored at 20 °C until use (no more than one month). Tissues presenting a thickness between 500 and 700 μm were used in the assays. Prior to use, the tissue was thawed in PBS pH 7.4 for, at least, 30 min.

2.2.1.2 TST permeation/retention through the human skin

Prehydrated human skin membranes were fixed between the donor and receptor chambers of the Franz-type diffusion cell, which present a diffusional area of 1.77 cm^2. The receptor chamber was filled with 10 mL of a PBS/propylene glycol solution (50/50, v/v) and kept at 37ºC under constant magnetic stirring at 750 rpm. Propylene glycol was selected for the receptor phase aiming to maintain a skin condition. The donor chamber was filled with 1% (w/w) of TST in propylene glycol (control solution) or this solution in presence of 10% (w/w/w) of each terpene (carvacrol, α-bisabolol and menthol). Different sampling time intervals were considered to define the experimental time (1, 2, 4, 6, 8, 12, 18 e 24 h or 8, 10, 12, 16, 18, 24, 30 and 36 h). After each aliquot withdrawal, fresh solution was added to keep constant the same volume in receptor phase. After 24 or 36 h, tissues were removed from diffusion chambers, cleaned using a cotton swab embedded with Milli-Q water and the epidermis was separated from the dermis with a cutting blade. The TST was extracted from the tissues by using an acetonitrile/PBS buffer mixture (70/30 v/v), followed of an ultrasound treatment for 30 min. Finally, the mixture solvent/tissue was centrifuged at 14,000 rpm for 5 min for protein precipitation. The content of TST was determined by HPLC for samples obtained from the receptor fluid and skin layers membranes.

The steady-state flux (J_{ss}) was calculated from the linear slope of the cumulative amount of TST permeated *versus* time. The permeability coefficient (P) was determined by the relation between Js and the initial donor concentration ($P = J_{ss}/C_d$) and lag time by the intercept of the regression line with the abscissa.

The expected testosterone plasma concentration (C_{ss}) was calculated by the following equation [10]:

$$C_{ss} = J_{ss} * A/CL \quad \text{(Equation 1)}$$

Where: J_{ss} is the permeation flux; A is the application area (40 cm^2), and CL represents the total testosterone body clearance, which is 54.33 L/h [11].

2.2.2 HPLC analysis

All the chromatographic separations were performed in an Agilent 1200 series HPLC system (Waldbronn, Germany) equipped with online degasser, quaternary pump, autosampler and diode array detector. The separation was performed in a Zorbax Eclipse Plus C18 column (100 x 4.6 mm ID, 3.5 µm particle size; Agilent, USA) at 30 °C. A isocratic elution of methanol and water (85/15, v/v) was considered as mobile phase. The flow rate and injection volume were 0.75 mL min^{-1} and 10 µL, respectively. The detector wavelength was set at 240 nm. The Open Lab software was used for data acquisition and treatment. A calibration curve in the range of 0.01 to 100 µg mL^{-1} was prepared in acetonitrile and water (70/30, v/v) from appropriate dilutions of stock solution. The calibration curve was constructed using peak area *versus* TST concentration ($y = 42.6x + 12.1$; $R^2 = 0.999$).

2.2.3 Mechanistic studies

2.2.3.1 DSC analysis

Thermal analyses were performed in a DSC-60 equipment (Shimadzu, Kyoto, Japan). Firstly, the stratum corneum (SC) was carefully separated from other skin layers and divided into sections of approximately 2 mg with a scalpel. SC samples were immersed in propylene glycol (control solution) containing 1% (w/w) of TST or in this solution in presence of 10% (w/w/w) of each terpene (carvacrol, α-bisabolol and menthol). Non-treated tissue (control) was immersed in a PBS solution. The period of exposure was similar to that of the permeation assay. The treated samples and control (untreated tissues) were sealed in aluminum hermetic pans and heated from 30 to

200 °C at a 5 °C/min rate in a nitrogen atmosphere. Changes in the enthalpy (ΔH) and corresponding mean transition temperature (*Tm*) values were monitored to understand lipid fluidization behavior or extraction processes as well as protein denaturation on the SC.

2.2.3.2 FT-IR spectral analysis

The SC was carefully separated from other skin layers and divided into sections of approximately 20 mg with a scalpel. SC samples were immersed in propylene glycol (control solution) containing 1% (w/w) of TST or in this solution in presence of 10% (w/w/w) of each terpene (carvacrol, α-bisabolol and menthol). Non-treated tissue (control) was immersed in a PBS solution. The period of exposure was similar to that of the permeation assay. FT-IR spectra of each sample were recorded in a frequency range from 600 to 4,000 cm^{-1} on a Perkin-Elmer Frontier equipment (Waltham, USA), at room temperature, with the collection of 20 scans at a resolution of 4 cm^{-1}. Data were shown as the mean spectrum of three different samples for each treatment.

2.2.4 Statistical analysis

The OriginPro 9 software was used for data analysis. One-way ANOVA followed by Tukey's post-hoc test was selected for analysis of the differences between groups/treatments.

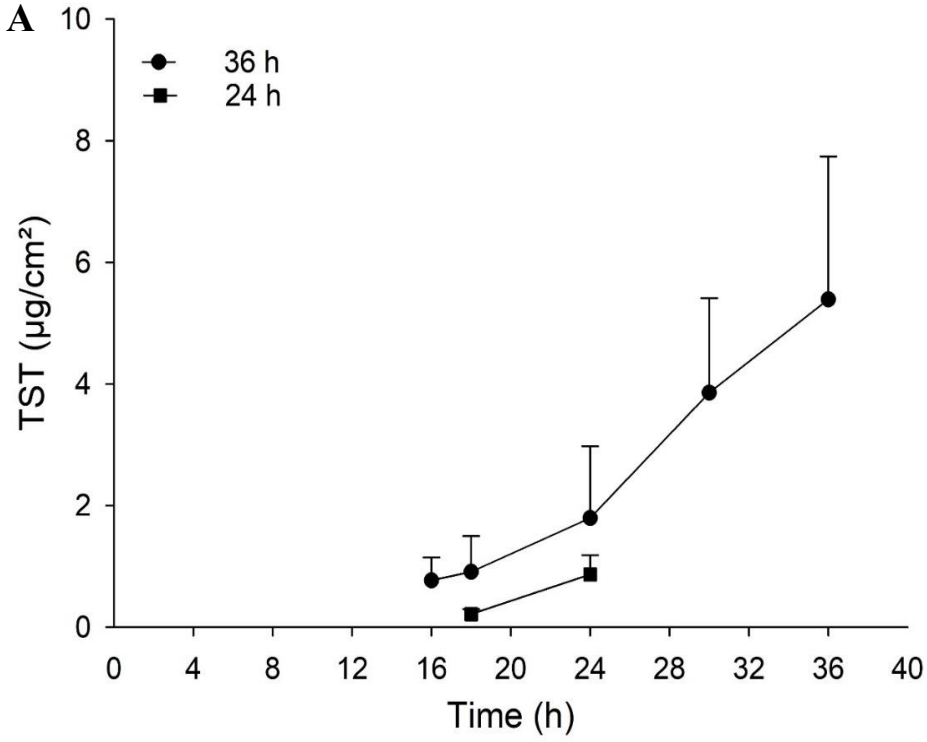
3. RESULTS

3.1. Ex vivo permeation studies

Once the TST has low aqueous solubility (24 μg mL^{-1}), a theoretical analysis of its solubility in different media was performed to define the composition of the receptor and donor fluids. The TST exhibits a solubility of 14.86 and 133.00 mg mL^{-1} in propylene glycol (PG) and ethanol, respectively (typical co-solvent often used in permeation assays) [12]. Although the hormone is more soluble in ethanol, PG was selected as solubilizing agents of TST due to the lower skin toxicity [13,14]. Moreover, studies have shown that a greatly increased solubilization of the TST in the donor phase of diffusion cells can negatively impact its transport rate. In a study testing 0.22, 6.31 and 5.14 mM of TST in only PBS, ethanol/PBS and propylene glycol/PBS, permeation coefficient values through equine skin were 6.82, 1.59 and 2.04 x 10^{-3} cm/h, respectively [15]. In another study with different concentrations of ethanol in water and

TST, lower transport rate through rat skin was found for increasing amounts of ethanol and TST [8].

The first part of the study was focused on determining the experimental time for the permeation assay. No terpene was considered in this step, only the control solution (TST in propylene glycol). This analysis is remarkable considering that the TST presents low transport rate and high affinity for the skin as already mentioned [16]. The *ex vivo* permeation assays were performed up to 24 and 36 h and different sampling time intervals were selected as described in method section. Interestingly, TST was quantified with precision only from 18 and 16 h for assays performed within the 24 and 36 h, respectively (Figure 2A). Once various samplings are carried out at the initial time intervals in the 24-hour assay, the receptor medium is diluted after the addition of fresh buffer. For this reason, TST was quantified appropriately later in the shorter duration assay (24 h). Based on this result, the first sample from 36 h assay was taken only 12 h after the start of the test. In this new time interval, TST was quantified with precision in different times and thus a more reliable permeation flux and profile were achieved. In cutaneous retention analysis, a two to threefold increase in amount of TST retained was found in both tissue layers (epidermis and dermis) when the assay time ranged from 24 to 36 h (Figure 2B). Therefore, 36 h was selected as experimental time to evaluate the effect of terpenes on TST permeation.



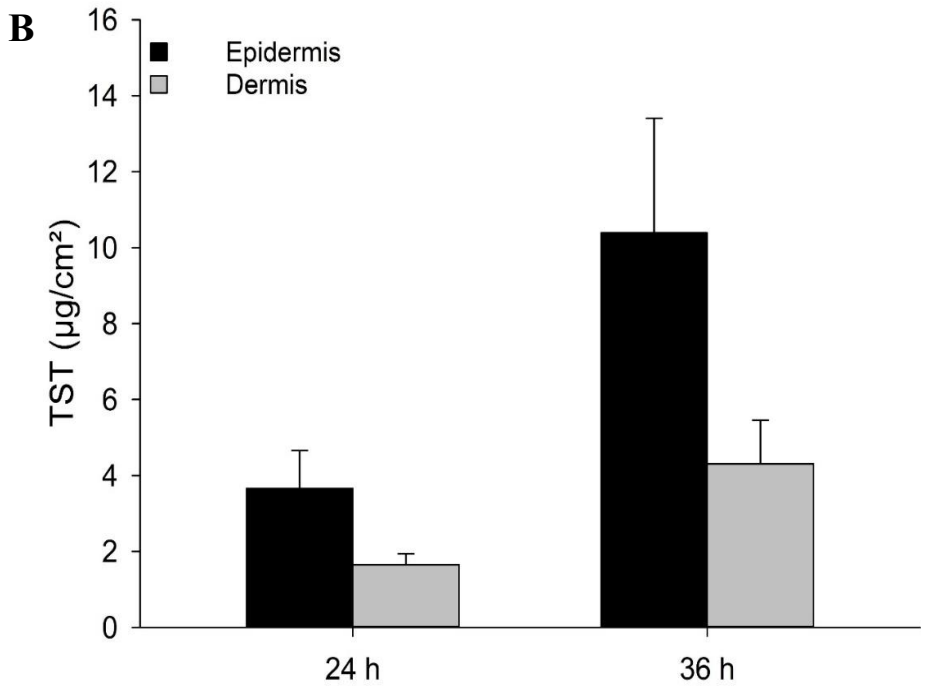


Figure 2. Cutaneous permeation (A) and retention (B) of testosterone through the human skin after 24 and 36 h (n=6).

The permeation of TST across human skin was enhanced in the presence of the terpenes (Fig. 3) compared to the control solution (1% w/w of TST in PG). In addition, the time required to TST achieve a steady-state flux (lag time) was approximately 1.6-fold shorter for carvacrol-treated skin compared to control-treated tissue (Table 1), suggesting a faster onset therapeutic activity in the presence of this terpene. The treatment with carvacrol increased the permeation flux and permeation coefficient of the TST in 7.6-fold compared to the control (Table 1). This terpene also significantly increased the amount of TST accumulated in the receptor chamber at 36 h (47.08 ± 16.79 $\mu g.cm^{-2}$) in relation to menthol (14.68 ± 9.39 $\mu g.cm^{-2}$). On the other hand, no significant difference (ANOVA/Tukey test, $p>0.05$) was observed between carvacrol and α-bisabolol (29.20 ± 11.85 $\mu g.cm^{-2}$).

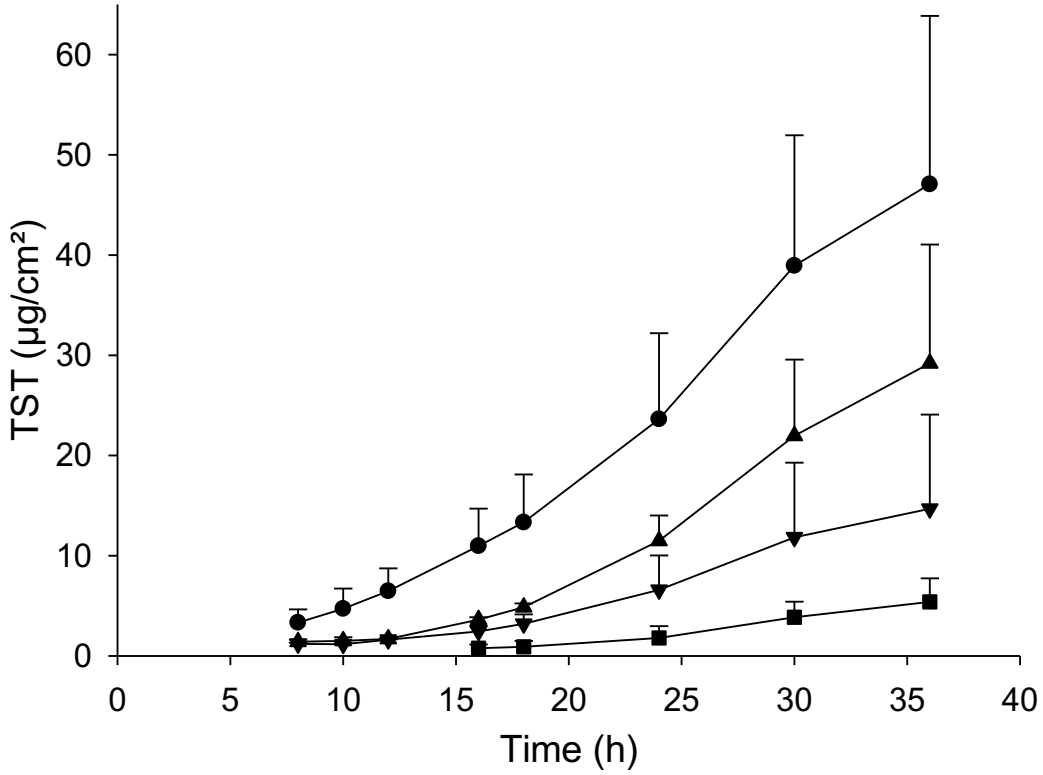


Figure 3. Skin permeation profile of testosterone in the presence of carvacrol (●), α-bisabolol (▲) and menthol (▼). TST in propylene glycol was used as control (■) (*n*=6).

Table 1. Permeation parameters of testosterone (TST) alone (control solution) and in the presence of terpenes (n=6).

Treatment	J_{ss} (µg.cm^{-2}.h)	P (cm.h^{-1} x 10^{-3})	Lag time (h)	Enhancement ratio (ER)
Control	0.215 ± 0.03	0.022 ± 0.003	12.99 ± 2.46	-
Carvacrol	1.638 ± 0.57*	0.164 ± 0.060*	7.95 ± 0.24*	7.61
α-bisabolol	1.072 ± 0.43	0.107 ± 0.043	11.65 ± 1.97	5.10
Menthol	0.542 ± 0.27	0.0542 ± 0.027	11.42 ± 3.35	2.52

*Statistically different compared to control (ANOVA/Tukey test, p <0.05).

For the obtained maximum permeation flux (J_{ss} = 1.638 µg/cm^2.h), the expected TST plasma concentration is 120.59 ng/dL. Although this concentration is outside the therapeutic range, which is 300-1000 ng/dL [17,18], it is important to mention that patients with hypogonadism already have a certain physiological concentration of the hormone (even if reduced). In addition, the absence of blood flow in *ex vivo* assays

hinders the permeation flux of active compounds through dermis layer. Therefore, the real permeation rates of TST should be higher in relation to the values found in *ex vivo* experiments. Changes in TST and terpene concentration as well as combination of CAEs could be alternatively considered.

Retention results of TST in the epidermis layer are in accordance with permeation data (Fig. 4). The treatment with carvacrol provided a significantly higher TST retention in the epidermis (305.20 ± 45.00 $\mu g.g^{-1}$) than control (120.60 ± 48.20 $\mu g.g^{-1}$) and menthol (119.20 ± 56.80 $\mu g.g^{-1}$); however, no significant difference (Tukey test, p <0.05) was found compared to α-bisabolol (249.50 ± 48.90 $\mu g.g^{-1}$). Once TST have more affinity for epidermis (more lipophilic skin layer), less amount of this hormone was found in the dermis. The amount of TST accumulated in dermis (Figure 4) after treatment with terpenes (carvacrol, 77.70 ± 21.20; α-bisabolol, 89.70 ± 14.20; menthol 29.90 ± 6.80) was not statistically different from the control (64.00 ± 4.60 $\mu g.g^{-1}$).

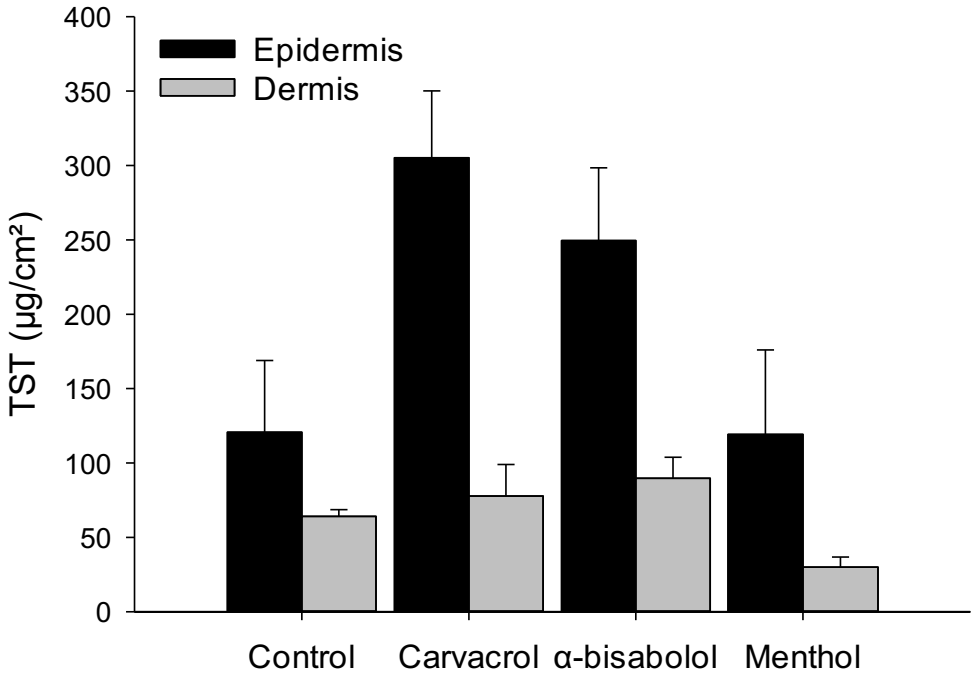


Figure 4. Retention of testosterone (TST) in the epidermis and dermis layers after treatment with terpenes. Control: 1% TST (w/w) in propylene glycol (n=6).

3.2 FT-IR analysis

Spectroscopic studies were considered to evaluate the effect of absorption enhancers on the biophysical properties of the stratum corneum (SC), particularly interactions with lipid or protein domains. The FT-IR spectrum of untreated SC (control) showed various bands associated to molecular vibrations of lipids (nonpolar) and proteins (polar) (Fig. 5). C-H stretching vibrations primarily associated with the lipid alkyl chains are represented by bands between 2,800 and 3,000 cm^{-1}. The bands at 2,920 and 2,850 cm^{-1}, in turn, are associated to the asymmetric CH_2 and symmetric CH_2 vibrations of long chain hydrocarbons of lipids, respectively. Asymmetric and symmetric CH_3 vibrations appear at 2,955 and 2,870 cm^{-1}, respectively [19]. Reduction in the intensity or significant shifts in these bands suggest disorder of the hydrocarbon chains or SC lipid domains [20]. Conversely, strong bands at 1,650 and 1,550 cm^{-1} are related to the amide I and amide II stretching vibrations of SC proteins, respectively [21]. The amide I bands arises from C=O stretching vibration and the amide II bands from C-N bending vibration. Both absorption bands are sensitive to hydrogen bonding interactions [22]. The amide I band represents various secondary structures of keratin.

The absorption band between 1,100 and 900 cm^{-1} is attributed to stretching vibrations of alcoholic C-O in C-O-H bonds as well as asymmetric and symmetric C-O-C bonds of propylene glycol [23]. A strong absorption band was identified close to 1,000 cm^{-1}. The presence of this agent in SC samples containing terpenes led to a reduction in the intensity of the bands in region between 3,000 and 3,600 cm^{-1}, which may be associated to its dehydration effect [24]. PG reduces the bound water content due to changes in the protein domains [25]. It probably works by solvating α-keratin and occupying hydrogen-bonding sites [26]. In fact, SC samples treated only with PG showed broader and lower band intensity at 1,650 and 1,550 cm^{-1} (amide region) compared to the control (untreated tissue), which suggest more disorder in protein domains. Menthol, in turn, resulted in less disorder in polar domains than carvacrol and α-bisabolol (carvacrol appears to have been the most effective). Unlike the SC sample containing menthol, the treatment with carvacrol and α-bisabolol led to broader and lower-intensity absorption bands at 2,920 and 2,850 cm^{-1}. This event indicates an increased translational movement or mobility of lipid acyl chains [24]. In summary, more changes in skin were showed after treatment with carvacrol.

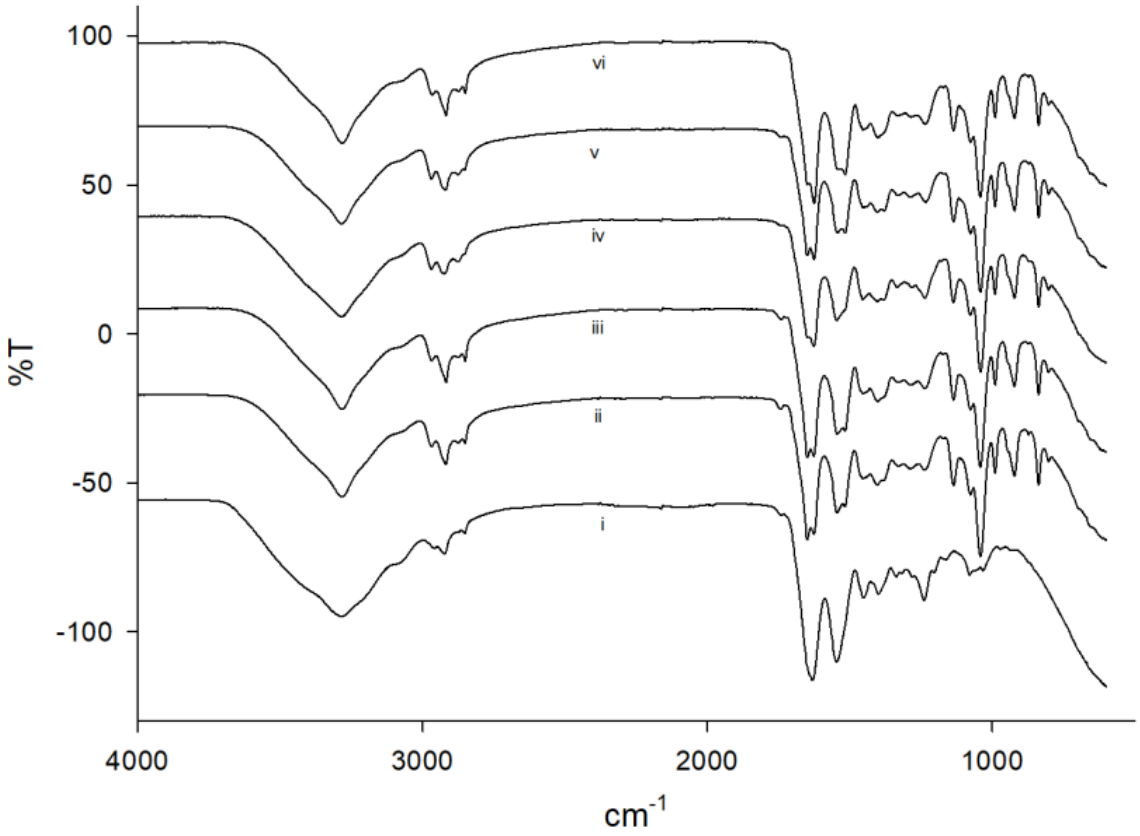


Figure 5. FT-IR spectra of untreated stratum corneum (i) and tissue treated only with propylene glycol (ii), TST in propylene glycol (iii); TST in propylene glycol and carvacrol (iv); TST in propylene glycol and α-bisabolol (v) and TST in propylene glycol and menthol (vi). (n = 2).

3.3 DSC analysis

DSC thermograms of SC treated with the tested absorption enhancers were evaluated by comparing T_m and ΔH of endotherms. The shift in T_m to a lower temperature indicates a disruption of the lipid bilayer whereas the reduction in ΔH values suggest fluidization of lipid bilayers [27].

Lipids extracted from the SC intercellular region show a single transition close to 65 °C [28]. As the SC treated with different solutions (PG alone or in presence of absorption enhancers) was dried with paper towel at room conditions before DSC analysis, extracted intercellular lipids were not identified in endotherms. In other words, the extracted lipids present in liquid medium are removed after the drying step and the amount of lipid available on the tissue would be not sufficient to generate an endotherm event (or significant signal in DSC analysis). This procedure was considered to minimize the PG effect in thermal analysis studies. A slight perturbation in the baseline

was found for SC treated with carvacrol at this temperature, but even more analyses should be performed to confirm this assumption.

The thermal transition close to 80 °C is found when lipids passing from the gel to the liquid-crystalline state. In this situation, lipids are associated with proteins (not more in a free form), probably as a lipid-protein complex [29]. Protein-lipid combinations often result in greater lipid ordering, which shift T_m to a higher temperature value compared to alone lipids [28]. SC treated with menthol and carvacrol showed a slight reduction in T_m value when compared to the control, suggesting a greater disruption of the lipid bilayer. Carvacrol and α-bisabolol-treated SC presented lower ΔH values than menthol-treated SC.

Transitions at temperatures above 90 ºC are often related to irreversible protein denaturation and have been detected in the delipidized SC matrices [30,31]. Therefore, this event could be detected in tissue samples where lipid extraction is found. SC samples treated with propylene glycol presented an event of protein denaturation close to 150 ºC, except the tissue that included menthol as absorption enhancer. Endothermic events in this temperature were more evident for the SC sample prepared with carvacrol and α-bisabolol, which may be associated with an increased delipidization provided by these absorption enhancers. As suggested in the FT-IR analyzes, both carvacrol and α-bisabolol interact more strongly with lipid domains than menthol. In addition, changes in hydration degree may affect relative peak heights. High temperature transitions (T>90 °C) are much more emphasized in the less hydrated lipids [31]. PG would reduce the SC hydration, making these endotherm events more evident. For the control, lipid-protein interactions are preserved and thus endotherm events associated with proteins (e.g., keratin) are not observed.

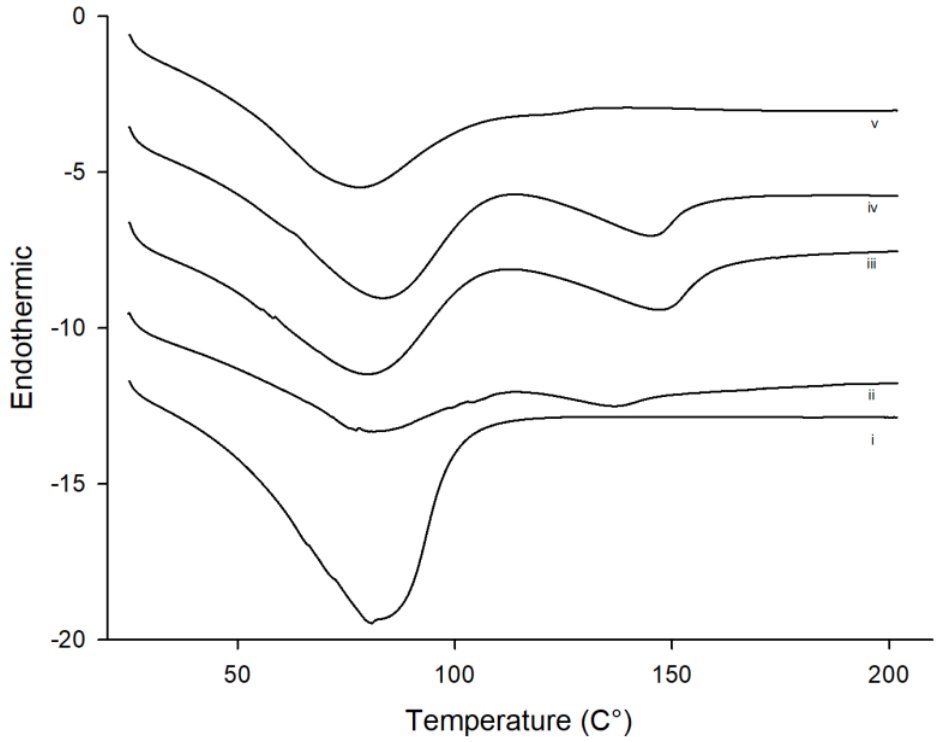


Figure 6. Thermograms of untreated stratum corneum (i), and stratum corneum treated with TST in propylene glycol (ii); TST in propylene glycol and carvacrol (iii); TST in propylene glycol and α-bisabolol (iv) and TST in propylene glycol and menthol (v). (n = 2).

4. DISCUSSION

Natural terpenes have attracted attention during the development of new pharmaceutical formulations due to permeation enhancement effect and low skin irritation. These agents have shown to be effective for both lipophilic and hydrophilic drug groups. They can fluidize and/or extract the SC lipids, weakening the skin barrier properties. This compound class is also included in the list of Generally Recognized As Safe (GRAS) agents issued by the US Food and Drug Administration [9].

Although the use of the menthol as the chemical absorption enhancer (CAE) in TST-based transdermal systems is already reported in the literature [32], the other two terpenes selected in this study had not yet been considered in transdermal systems with this drug. In a previous study carried out by our research team, menthol showed to be less active than the limonene monoterpene in enhancing drug permeation. Differences in degree of disorder in lipid bilayers would explain these findings [27]. For this reason, in this new study, a comparison among three terpenes was considered taking in account

the action differences. In this same way, menthol showed to be the least effective terpene as CAE. An enhancement ratio (ER) of 2.52 was obtained in our study, which is in agreement with the study of Kaplun-Frischoff and coworkers (1997). The authors found a 2.6-fold increase in the flux value of TST through skin after a treatment with menthol. The formation of a eutectic mixture between TST and menthol as well as changes in barrier properties could explain these results [32]. Although carvacrol and menthol are structurally similar and present quite similar physicochemical properties (boiling point, log P and molecular weight; Table 2), different absorption rates for the TST were found after treatment with these CAEs. Spectroscopic and thermal analyses suggest that carvacrol showed greater affinity for lipid domains than menthol. As TST is preferentialy transported by the transcelullar/intercellular passive route due to its lipophilic nature [33,34], CAEs that modify these domains are more effective in increasing its permeation flux. The presence of the aromatic ring and phenolic OH in carvacrol also contribute to a greater chemical reactivity than menthol, which present a hydroxyl group linked to the cycloalkane (a less reactive chemical group). Therefore, a greater interaction with organic molecules (skin constituents) was expected for the carvacrol.

A similar interaction/specificity for skin lipid and protein domains was observed for carvacrol and α-bisabolol in spectroscopic and thermal analyses. On the other hand, the permeation flux of TST was higher in the presence of the carvacrol. A detailed analysis of the physicochemical properties of terpenes can contribute to explain these differences. α-bisabolol presents a higher molar mass and log P. The high log P value leads to a preferential interaction with the most superficial layers of the skin and its higher molar mass compared to carvacrol reduces the diffusion through the skin. In fact, TST presented a slower diffusion (lower lag time) through skin in the presence of α-bisabolol. Furthermore, α-bisabolol has a higher melting point and boiling point than carvacrol. Higher melting and boiling points indicate stronger noncovalent intermolecular forces. Consequently, lower degree of interaction with other molecules (e.g., skin constituents) is expected. The boiling point of a terpene have been inversely related to its skin penetration enhancement effects [9]. Therefore, interactions among competitive hydrogen bonding of terpenes and skin ceramides are facilitated when the terpene presents a low boiling point.

Table 2. Classification and properties of terpenes.

Terpene	Classification	Molar mass (g mol^{-1})	Melting point (^oC)	Boiling point (^oC)	Log P
α-bisabolol	Sesquiterpene	222.37	25	314.5	5.07
Carvacrol	Monoterpene	150.22	3.5	237.7	3.28
Menthol	Monoterpene	156.27	43	215.4	3.20

Chen *et al.* (2016) and Kang *et al.* (2013)

Concerning the TST retention data, the greater affinity of the hormone for skin lipophilic structures can explain its higher retention in the epidermis compared to the dermis layer for all treatments. The presence of androgen receptors in the skin structures such as sebocytes, hair follicle, dermal papilla and keratinocytes [36] may contribute to an increased concentration of TST in the superficial layers.

Terpenes have also been considered in permeation studies with other hormones. Songkro et al. (2009), for example, evaluated the impact of carvone, carvacrol and menthol on the permeation rate of luteinizing hormone releasing hormone (LHRH) through newborn pig. Unlike the TST, LHRH is a hydrophilic molecule with a large molecular weight (1,182 g mol^{-1}), which limits its transport via the passive route. Terpenes were able to improve the cutaneous absorption of LHRH. Similar to our study, carvacrol was also the most effective CAE [37].

In addition to the effect of enhancing percutaneous absorption, the selection of carvacrol for transdermal formulations with TST leads to several other advantages various. In assays with rats, carvacrol demonstrated a significant antioxidant effect and improved sperm quality parameters such as motility, concentration, abnormal spermatozoa and live-dead sperm ratio [38]. This effect is particularly relevant in the case of hypogonadism. Due to the antibacterial and antifungal properties [39], carvacrol also could play a role as a formulation preservative. In this context, the irritating synthetic preservatives could be avoided or their use concentration could be reduced. Another interesting aspect is that this terpene does not affect the metabolization of TST via CYP3A4 [40], a common problem when natural materials are co-administered with synthetic drugs.

5. CONCLUSION

The terpenes carvacrol, α-bisabolol and menthol showed to be effective in enhancing the permeation of TST through human skin, resulting in ER values of 7.61, 5.10 and 2.52, respectively. Carvacrol interacts more strongly with lipid domains than other terpenes, which result in a greater disorder at these sites. As the TST is preferentially transported through the skin by the passive diffusion, a greater lipid disorder has direct impact on the permeation flux. In addition to a permeation-enhancing effect, carvacrol is non-irritating, presents antioxidant, antifungal and antibacterial effect and may improve sperm quality parameters. Taken together, these aspects suggest that this terpene is an ideal candidate for transdermal delivery systems containing TST.

DECLARATION OF COMPETING INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors would like to thank Quality Control Laboratory (UFSC) for the technical support in DSC and FTIR analyses.

REFERENCES

[1] R. Neupane, S.H.S. Boddu, J. Renukuntla, R.J. Babu, A.K. Tiwari, Alternatives to Biological Skin in Permeation Studies: Current Trends and Possibilities, Pharmaceutics. 12 (2020) 152. https://doi.org/10.3390/pharmaceutics12020152.

[2] J.J. Shoskes, M.K. Wilson, M.L. Spinner, Pharmacology of testosterone replacement therapy preparations, Transl. Androl. Urol. 5 (2016) 834–843. https://doi.org/10.21037/tau.2016.07.10.

[3] N. Shahidi, A review of the chemistry, biological action, and clinical applications of anabolic-androgenic steroids, Clin. Ther. 23 (2001) 1355–1390. https://doi.org/10.1016/S0149-2918(01)80114-4.

[4] C. Koch, M.I. Ullah, D. Riche, Transdermal testosterone replacement therapy in men, Drug Des. Devel. Ther. (2014) 101. https://doi.org/10.2147/DDDT.S43475.

[5] B.M. Magnusson, S.E. Cross, G. Winckle, M.S. Roberts, Percutaneous Absorption of Steroids: Determination of in vitro Permeability and Tissue Reservoir Characteristics in Human Skin Layers, Skin Pharmacol. Physiol. 19 (2006) 336–342. https://doi.org/10.1159/000095254.

[6] G. Pelletier, L. Ren, Localization of sex steroid receptors in human skin, Histol. Histopathol. 19 (2004) 629–636. https://doi.org/10.14670/HH-19.629.

[7] D. Pabla, H. Zia, A comparative permeation/release study of different testosterone gel formulations, Drug Deliv. 14 (2007) 389–396. https://doi.org/10.1080/10717540701203075.

[8] M.K. Kim, H. Zhao, C.H. Lee, D.D. Kim, Formulation of a reservoir-type testosterone transdermal delivery system, Int. J. Pharm. 219 (2001) 51–59. https://doi.org/10.1016/S0378-5173(01)00631-7.

[9] J. Chen, Q.-D. Jiang, Y.-P. Chai, H. Zhang, P. Peng, X.-X. Yang, Natural Terpenes as Penetration Enhancers for Transdermal Drug Delivery, Molecules. 21 (2016) 1709. https://doi.org/10.3390/molecules21121709.

[10] D. Ainbinder, E. Touitou, Testosterone ethosomes for enhanced transdermal delivery, Drug Deliv. 12 (2005) 297–303.

[11] Q.-F. Xing, S. Lin, Y.W. Chien, Transdermal testosterone delivery in castrated Yucatan minipigs: pharmacokinetics and metabolism, J. Control. release. 52 (1998) 89–98.

[12] D. Karadzovska, J.D. Brooks, J.E. Riviere, Modeling the effect of experimental variables on the in vitro permeation of six model compounds across porcine skin, Int. J. Pharm. 443 (2013) 58–67. https://doi.org/10.1016/j.ijpharm.2013.01.002.

[13] J. Park, H. Lee, K. Park, Mixture Toxicity of Methylisothiazolinone and Propylene Glycol at a Maximum Concentration for Personal Care Products, Toxicol. Res. 34 (2018) 355–361. https://doi.org/10.5487/TR.2018.34.4.355.

[14] A.K. Levang, K. Zhao, J. Singh, Effect of ethanol/propylene glycol on the in

vitro percutaneous absorption of aspirin, biophysical changes and macroscopic barrier properties of the skin, Int. J. Pharm. 181 (1999) 255–263. https://doi.org/10.1016/S0378-5173(99)00055-1.

[15] P.C. Mills, Vehicle effects on the In vitro penetration of testosterone through equine skin, Vet. Res. Commun. 31 (2007) 227–233. https://doi.org/10.1007/s11259-006-3446-6.

[16] N. Esposto Biondo, D. Fretes Argenta, G. Schneider Rauber, T. Caon, How to define the experimental conditions of skin permeation assays for drugs presenting biopharmaceutical limitations? The experience with testosterone, Int. J. Pharm. 607 (2021) 120987. https://doi.org/10.1016/j.ijpharm.2021.120987.

[17] S. Basaria, A.S. Dobs, Hypogonadism and androgen replacement therapy in elderly men, Am. J. Med. 110 (2001) 563–572.

[18] W.J. Bremner, M. V. Vitiello, P.N. Prinz, Loss of Circadian Rhythmicity in Blood Testosterone Levels with Aging in Normal Men, J. Clin. Endocrinol. Metab. 56 (1983) 1278–1281. https://doi.org/10.1210/jcem-56-6-1278.

[19] M. Cotte, P. Dumas, M. Besnard, P. Tchoreloff, P. Walter, Synchrotron FT-IR microscopic study of chemical enhancers in transdermal drug delivery: example of fatty acids, J. Control. Release. 97 (2004) 269–281. https://doi.org/10.1016/j.jconrel.2004.03.014.

[20] E.C. Guillard, A. Tfayli, C. Laugel, A. Baillet-Guffroy, Molecular interactions of penetration enhancers within ceramides organization: A FTIR approach, Eur. J. Pharm. Sci. 36 (2009) 192–199. https://doi.org/10.1016/j.ejps.2008.10.010.

[21] I. Vashisth, A. Ahad, M. Aqil, S.P. Agarwal, Investigating the potential of essential oils as penetration enhancer for transdermal losartan delivery: Effectiveness and mechanism of action, Asian J. Pharm. Sci. 9 (2014) 260–267. https://doi.org/10.1016/j.ajps.2014.06.007.

[22] P. Garidel, Mid-FTIR-Microspectroscopy of stratum corneum single cells and stratum corneum tissue, Phys. Chem. Chem. Phys. 4 (2002) 5671–5677. https://doi.org/10.1039/b207478h.

[23] A. Pourfarzad, Z. Ahmadian, M.B. Habibi-Najafi, Interactions between polyols and wheat biopolymers in a bread model system fortified with inulin: A Fourier transform infrared study, Heliyon. 4 (2018) e01017. https://doi.org/10.1016/j.heliyon.2018.e01017.

[24] R. Panchagnula, P.S. Salve, N.S. Thomas, A.K. Jain, P. Ramarao, Transdermal delivery of naloxone: Effect of water, propylene glycol, ethanol and their binary combinations on permeation through rat skin, Int. J. Pharm. 219 (2001) 95–105. https://doi.org/10.1016/S0378-5173(01)00634-2.

[25] D.K. Shah, S. Khandavilli, R. Panchagnula, Alteration of skin hydration and its barrier function by vehicle and permeation enhancers: A study using TGA, FTIR, TEWL and drug permeation as markers, Methods Find. Exp. Clin. Pharmacol. 30 (2008) 499. https://doi.org/10.1358/mf.2008.30.7.1159653.

[26] B.W. Barry, Mode of action of penetration enhancers in human skin, J. Control. Release. 6 (1987) 85–97. https://doi.org/10.1016/0168-3659(87)90066-6.

[27] T. Caon, C.E.M. Campos, C.M.O. Simões, M.A.S. Silva, Novel perspectives in the tuberculosis treatment: Administration of isoniazid through the skin, Int. J. Pharm. 494 (2015) 463–470. https://doi.org/10.1016/j.ijpharm.2015.08.067.

[28] G.M. Golden, D.B. Guzek, R.R. Harris, J.E. McKie, R.O. Potts, Lipid Thermotropic Transitions in Human Stratum Corneum, J. Invest. Dermatol. 86 (1986) 255–259. https://doi.org/10.1111/1523-1747.ep12285373.

[29] R. Pouliot, L. Germain, F.A. Auger, N. Tremblay, J. Juhasz, Physical characterization of the stratum corneum of an in vitro human skin equivalent produced by tissue engineering and its comparison with normal human skin by ATR-FTIR spectroscopy and thermal analysis (DSC), Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 1439 (1999) 341–352. https://doi.org/10.1016/S1388-1981(99)00086-4.

[30] C.L. Silva, S.C.C. Nunes, M.E.S. Eusébio, A.A.C.C. Pais, J.J.S. Sousa, Thermal Behaviour of Human Stratum Corneum. A differential scanning calorimetry study at high scanning rates, Skin Pharmacol. Physiol. 19 (2006) 132–139. https://doi.org/10.1159/000092593.

[31] C.L. Silva, S.C.C. Nunes, M.E.S. Eusébio, J.J.S. Sousa, A.A.C.C. Pais, Study of human stratum corneum and extracted lipids by thermomicroscopy and DSC, Chem. Phys. Lipids. 140 (2006) 36–47. https://doi.org/10.1016/j.chemphyslip.2006.01.004.

[32] Y. Kaplun-Frischoff, E. Touitou, Testosterone skin permeation enhancement by menthol through formation of eutectic with drug and interaction with skin lipids, J. Pharm. Sci. 86 (1997) 1394–1399. https://doi.org/10.1021/js9701465.

[33] I. Iachina, I.E. Antonescu, J. Dreier, J.A. Sørensen, J.R. Brewer, The nanoscopic molecular pathway through human skin, Biochim. Biophys. Acta - Gen. Subj. 1863 (2019) 1226–1233. https://doi.org/10.1016/j.bbagen.2019.04.012.

[34] X.-Q. Niu, D.-P. Zhang, Q. Bian, X.-F. Feng, H. Li, Y.-F. Rao, Y.-M. Shen, F.-N. Geng, A.-R. Yuan, X.-Y. Ying, J.-Q. Gao, Mechanism investigation of ethosomes transdermal permeation, Int. J. Pharm. X. 1 (2019) 100027. https://doi.org/10.1016/j.ijpx.2019.100027.

[35] L. Kang, V.H. Kumar, P.F.C. Lim, H.H. Cheong, S.Y. Chan, Terpenes and Improvement of Transdermal Drug Delivery, in: Nat. Prod., Springer Berlin Heidelberg, Berlin, Heidelberg, 2013: p. 3757–3774. https://doi.org/10.1007/978-3-642-22144-6_160.

[36] R. Choudhry, M.B. Hodgins, T.H. Van der Kwast, A.O. Brinkmann, W.J.A. Boersma, Localization of androgen receptors in human skin by immunohistochemistry: implications for the hormonal regulation of hair growth, sebaceous glands and sweat glands, J. Endocrinol. 133 (1992) 467-NP. https://doi.org/10.1677/joe.0.1330467.

[37] S. Songkro, T. Rades, G. Becket, Effects of some terpenes on the in vitro permeation of LHRH through newborn pig skin, Die Pharm. Int. J. Pharm. Sci. 64 (2009) 110–115.

[38] M. Güvenç, M. Cellat, İ. Gökçek, İ. Yavaş, Ş. Yurdagül Özsoy, Effects of thymol

and carvacrol on sperm quality and oxidant/antioxidant balance in rats, Arch. Physiol. Biochem. 125 (2019) 396–403. https://doi.org/10.1080/13813455.2018.1476979.

[39] A. Marchese, I.E. Orhan, M. Daglia, R. Barbieri, A. Di Lorenzo, S.F. Nabavi, O. Gortzi, M. Izadi, S.M. Nabavi, Antibacterial and antifungal activities of thymol: A brief review of the literature, Food Chem. 210 (2016) 402–414. https://doi.org/10.1016/j.foodchem.2016.04.111.

[40] Y. Li, G.D. Kashangaki, F.. Yl, Interactions of testosterone and carbamazepine with carvacrol mediated by CYP3A4, Chinese J. Pharmacol. Toxicol. 34 (2020) 605–609.

5. CAPÍTULO IV – UMA ANÁLISE COMPARATIVA DE MODELOS DE PELES BIOLÓGICAS E SINTÉTICAS PARA ESTUDOS DE TRANSPORTE DE FÁRMACOS

Este capítulo trata da utilização de modelos alternativos de peles em estudos de permeação de fármacos. Maior ênfase foi dada aos modelos de pele reconstruída e os de pele sintética, mas a pele de animais também foi incluída na análise. Uma maneira racional de comparar o desempenho de diferentes modelos biológicos e não biológicos de pele é selecionar estudos de permeação realizados com moléculas similares. Por este motivo, testosterona e cafeína foram selecionados como modelo de fármaco lipofílico e hidrofílico, respectivamente. O produto deste capítulo é um artigo científico, que será submetido para apreciação no periódico *International Journal of Pharmaceutics Sciences*.

5.1. ARTIGO CIENTÍFICO III

A COMPARATIVE ANALYSIS OF BIOLOGICAL AND SYNTHETIC SKIN MODELS FOR DRUG TRANSPORT STUDIES

Nicole Esposto Biondo; Débora Fretes Argenta; Thiago Caon[#]

Postgraduate Program in Pharmacy, Federal University of Santa Catarina, Campus Reitor João David Ferreira Lima, s/n - Trindade, Florianópolis, SC 88040-900, Brazil.

[#]Corresponding author: thiagocaon@gmail.com; caon.thiago@ufsc.br

To be submitted in the International Journal of Pharmaceutical Sciences

ABSTRACT

Ethical restrictions as well as practical or economic issues related to use of animal and human skin has been the main reason for the increase in the number of investigations with alternative models. Reconstructed skin models, for example, have been useful to evaluate the *in vitro* toxicity of compounds; however, these models usually overestimate the amount of drug permeated due to impaired barrier properties. In this review, two compounds presenting different physicochemical properties were selected to compare the performance of synthetic and biological skin models in transport studies. Advantages and limitations of each skin model are discussed in detail. Although synthetic and reconstructed skin models have shown to be useful in formulation optimization step, they present many limitations: (1) more permeation to drugs; (2) lack of follicular transport; (3) no metabolism in synthetic membranes; (4) differences in terms of lipid organization; (5) more affected by formulation constituents. Therefore, animal and human tissues should still be prioritized in drug transport studies until new advanced alternative models are purposed. Investigations of the impact of culture conditions on skin formation, in turn, bring perspectives related to development of unhealthy/injured skin models (an aspect that still deserves attention).

Keywords: synthetic skin; skin equivalents; permeation; testosterone; caffeine.

1. INTRODUCTION

The skin is a heterogeneous organ that present several drug administration sites and thus it may be targeted to treat different diseases (Abd et al., 2016). It presents various advantages over conventional administration routes (e.g., oral and intravenous). It avoids the first-pass metabolism, protects compounds from the degradation in gastrointestinal tract, allows a sustained release of drugs for extended periods due to a "reservoir" effect, may reduce toxicity and local irritation and still provides high patient compliance to treatment as it is a non-invasive administration route (Haq et al., 2018; Roberts et al., 2021; Yu et al., 2021). For this reason, pharmaceutical companies have often performed investigations aiming to reposition oral drugs to the transdermal route.

Cutaneous permeation assays represent a key point during the development of new cosmetic or transdermal products for human use (Planz et al., 2016; Yun et al., 2018). Appropriate quality control should also be performed to ensure batch-to-batch uniformity in the pharmaceutical industry. Clinical bioequivalence studies have been particularly considered in performance evaluation of new topical or transdermal products to ensure that an enough amount of drug achieves the target; however, these assays are highly costly, associated with high intra-subject variability and little sensitive to discriminate formulations presenting different composition (Abd et al., 2016).

In vitro/ex vivo permeation studies emerge as an alternative to evaluate the bioequivalence of transdermal dosage forms and for mechanistic investigations (Raney et al., 2015). Franz-type diffusion cells has been the most used *ex vivo* model for skin permeation studies, in which different membrane types (human, animal and synthetic) are sandwiched between a donor compartment and a receptor compartment (Haq et al., 2018; Küchler et al., 2013). Assays with human skin represent the gold standard; however, ethical, practical or economic reasons as well as tissue supply have limited its use (Esposto Biondo et al., 2021). Regulatory agencies and consumers have also pressured the industry to avoid experimentation with animal tissues in last decade, particularly in the cosmetic field (Abd et al., 2016). In this context, cell-culture skin models, synthetic or natural biomaterial-

based scaffolds have attracted the attention of researchers (Flaten et al., 2015). Although different reconstructed skin models have been already approved by OECD for toxicity assays (OECD, 2004a, 2004b), the use of these membrane models in drug absorption studies is still challenging as a barrier function similar to *in vivo* skin should be found (Van Gele et al., 2011). The advantages and disadvantages/limitations of each skin model are summarized in Table 1.

Table 1. Advantages and limitations of different skin models.

Model skin	Advantages	Limitations
Human	-Gold standard for permeation studies	-Ethical considerations; -Inter-individual variability; -Limited tissue availability.
Animal	-Animal models such as porcine skin present barrier properties similar to human skin; -Some animals (rat/mouse) present hairless species available to investigate the role of hair follicles in drug permeation.	-Rat/mouse model require ethical permission; -Large variability interspecies (e.g., Skin thickness and hair follicle density vary depending on the animal model).
Artificial/ synthetic membranes	-High correlation with *in-silico* models as mimic transcellular transport of drugs; -Application for screening purposes in early phases of drug development; -Reproducible; -Assays may be performed in a short time (short lag time); -Low cost.	-Poor barrier properties which result in increased absorption rate; -No effective for drugs that suffer skin metabolism; -No appendageal transport; -Model is highly sensitive to the incorporation of chemical absorption enhancers and surfactants.
Reconstructed skin model (cell cultures)	-Are effective for mechanistic investigations (e.g., skin metabolism, transport type); -Models with and without stratum corneum can be prepared; -More sensitive to the incorporation of chemical absorption enhancers and surfactants than human and animal tissues; -Reproducible; -Assays may be performed in a short time (short lag time).	-More permeable than human skin; -Time-consuming (long culture time); -No appendageal transport; -High cost.

A rational way to compare the performance of different biological and non-biological skin models is to select permeation studies performed with the same drug model. Therefore, *in vitro-ex vivo-in vivo* correlations can be more easily established. Caffeine (CF) is often used in skin permeation assays as a highly water-

soluble compound whereas the testosterone (TST) as a lipophilic drug (Abd et al., 2016; Neupane et al., 2020; Schreiber et al., 2005; van de Sandt et al., 2004). The log P values of CF (MM=194.19 g mol^{-1}) and TST (MM=288.4 g mol^{-1}) are -0.07 and 3.32, respectively. At 25°C, the solubility of CF and TST in water is 2.16×10^4 and 23.4 mg L^{-1}, respectively (Im et al., 2021). These different physicochemical properties impact on transport mechanisms through the skin. Hydrophobic drugs such as TST are preferentially transported through the "intracellular" pathway as they need to cross the lipid bilayer whereas "intercellular" pathway is preferentially used for hydrophilic compounds as CF. In this last situation, molecule diffusion around the cells occurs in a tortuous manner (Supe e Takudage, 2021) and a lipid disorder or fluidization is crucial for an efficient transport (Neupane et al., 2020). The "transappendageal" route (e.g. hair follicle, sebaceous glands), in turn, is more used for polar molecules that do not freely cross the stratum corneum (Dominguez et al., 2017).

CF and TST are also recommended as reference compounds by the OECD to validate the *in vitro/ex vivo* model used in investigation of cutaneous permeation of new products or drugs (OECD, 2000a, 2000b, 2000c). Therefore, the main objective of this literature review was comparing the performance of different biological and non-biologic membranes in cutaneous permeations studies considering these two drugs; understanding the advantages and limitations of each skin model. Studies performed with formulations of these compounds or free drug in presence of chemical absorption enhancers were not considered for this review.

2. HUMAN SKIN STRUCTURE

The human skin structure is composed of three main layers: epidermis, dermis and subcutaneous tissues (Figure 1). The thickness and composition of the skin could vary with age, anatomical site, race and gender (Roberts et al., 2021).

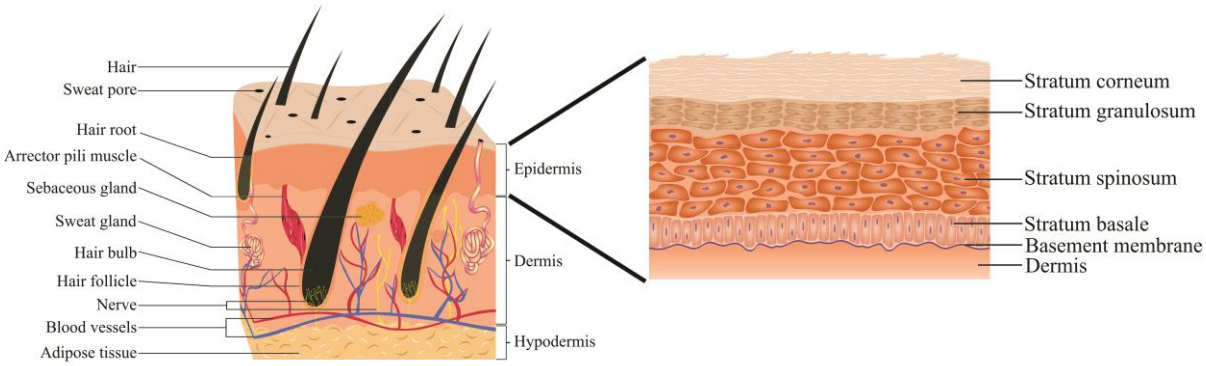


Figure 1. Skin structure (left) and main layers of the epidermis (right).

The major drug diffusion-limiting barrier in skin is represented by *stratum corneum* (SC), which is the outermost layer of epidermis. It is a highly hydrophobic layer of 10-20 μm, composed of 10-25 layers of stacked corneocytes, which are anucleated and devitalized cells. Corneocytes are embedded in a structurally well-organized matrix of intercellular lipids, which is referred as a "bricks-in-mortar" structure (Sjövall et al., 2018). The SC lipid matrix is dominated by three lipid classes (cholesterol, free fatty acids and ceramides), which are synthesized by lamellar bodies of keratinocytes during the differentiation process to corneocytes (Évora et al., 2021).

The viable epidermis (50-100 μm thick), underlying the SC, is a stratified epithelium composed of basal, spinous and granular cell layers. Each viable epidermis layer is defined by position, shape, morphology and state of differentiation of keratinocytes (Bouwstra, 2003). Keratinocytes proliferate in the basal layer and then suffer a differentiation process while migrating to the skin outermost layers (Évora et al., 2021). Many proteins and lipids are synthesized and expressed during keratinocyte maturation. Expressive changes in keratinocyte structure are found in the last differentiation stage, which lead to a programmed cell death process known as "cornification". In other words, viable cells are converted into chemically and physically resistant cornified squames called "corneocytes" (Bouwstra, 2003).

The dermis is the skin deeper layer with 1-2 mm thick, which allows the drug access to circulation system due to presence of blood vessels (Prausnitz e Langer, 2008). Unlike the epidermis, which is made up of dense keratinocytes, the dermis is mostly composed of an acellular component, the extracellular matrix (ECM). This structural feature explains why highly lipophilic drugs difficulty crossing the dermis. Collagen fibers, particularly collagen type I and III, represent the major constituent of the ECM (75% of the dry weight of skin). These proteins provide tensile strength and elasticity to the skin. Elastic fiber network, in turn, allows the skin return to its original shape after stretching or deformation. Although proteoglycans and glycosaminoglycans represent only 0.2% of the dry weight of the dermis, they are able to absorb water up to 1,000 times their weight. In this way, they play an important physiological role in maintaining skin hydration. The main

resident cell type of the dermis are the fibroblasts, which are involved in the production of ECM constituents (Shin et al., 2019).

The hypodermis or subcutaneous tissue, the innermost layer of the skin, is rich in adipocytes and play an important role in thermoregulation and maintenance of the mechanical properties. In addition, it has supported keratinocyte and fibroblast proliferation and regulate cycling of hair follicles (Zimoch et al., 2021). During drug transport assays using human or animal tissues, hypodermis is often removed to prevent drug absorption data from being underestimated.

Pilosebaceous units have been the main appendages associated with drug transport through the skin. In these structures, each hair follicle is coupled with one or more flask-shaped sebaceous glands. Once the density and size of hair follicles vary depending on the region of the body, this aspect should be carefully monitored during drug transport studies. The size of the follicular orifice and presence of sebum in follicular space also affect the absorbed drug amount (Verma et al., 2016).

3. TRANSPORT STUDIES IN HUMAN SKIN

Although *ex vivo* assays cannot fully reproduce *in vivo* conditions, particularly with respect to metabolism, distribution, and blood supply, an important advantage of this model is that experimental conditions can be controlled more precisely (Brain et al., 2002). *Ex vivo-in vivo* correlations have been successfully achieved under rigorous or controlled experimental conditions, which is of particular interest to regulatory agencies seeking standardized or robust methods (Abd et al., 2016).

Diffusion cells, particularly the Franz-type cells, represent the most traditional method to evaluate the *ex vivo* permeation of drugs, which consists of donor and acceptor chamber sandwiched by a membrane/tissue (Moser et al., 2001). Excised human skin is often obtained from plastic surgery or cadavers. In both situations, appropriate ethical approval is required to use the tissue. Abdominal, breast, or back skin are routinely used because the larger tissue area compared to other body regions (Abd et al., 2016).

"Full thickness skin" is prepared by removal of subcutaneous fat, separating dermis and epidermis (Cross et al., 2003). The use of membranes with a thickness of more than 1.0 mm (epidermis and dermis) is not recommended once the absorption of lipophilic drugs may be reduced or even avoided (van de Sandt et al., 2004). For this reason, dermatomes have been routinely considered to reduce the thickness tissue to approximately 500-750 μm (Abd et al., 2016). Manual tissue preparation can also be performed, but their thickness should be carefully monitored to reduce experimental variability. Once the skin is obtained, its integrity may be evaluated through transepithelial electrical resistance, transepidermal water loss and permeation of tritiated water measurements or histological evaluation (Esposto Biondo et al., 2021).

The *in vitro* absorption of CF and TST through human skin was evaluated in a multicenter comparison study that considered data from the 9 laboratories. A common protocol was defined as well as three independent assays for each tested compound. The absorption of CF and TST through human skin was similar, presenting mean maximum absorption rates of 2.24 ± 1.43 and 1.63 ± 1.94 $\mu g/cm^2/h$, respectively. In 7 out of 9 laboratories, the maximum absorption rates of CF were ranked higher than TST. Although laboratories used different diffusion cell apparatus, this variable did not affect the transport rate of these compounds significantly. On the other hand, skin thickness showed to affect the absorption of TST. Thinner and dermatomed tissues resulted in a maximum absorption rate. This parameter did not affect the permeation rate of CF significantly (van de Sandt et al., 2004). Atrux-Tallau et al. (2007) also found a similar permeation flux for CF through the dermatomed human skin and heat-separated epidermal tissues (Atrux-Tallau et al., 2007).

In another study performed with CF and TST in a flow-through cells and human skin, similar findings related to impact of skin thickness on permeation of TST were found. No significant differences in maximum permeation flux values for CF were observed with thickness variations. TST, in turn, showed a permeation flux approximately 10-fold higher in the thinner skin (Wilkinson et al., 2006).

In summary, these literature studies sustain the hypothesis that skin thickness needs to be reduced prior to permeation assays with lipophilic drugs to obtain results closer to physiological situation.

4. ANIMAL SKIN MODELS

Animal skin models differs from the human skin with respect to number of hair follices, lipid content and composition as well as anatomical or morphological aspects (Van Gele et al., 2011) (Table 2). The porcine skin has been considered the main substitute for human skin due to anatomical, physiological and biochemical similarity (Barbero e Frasch, 2009; Simon e Maibach, 2000). Both tissues are characterized by a thick epidermis, a dermis presenting a well-differentiated papillary body and a large content of elastic tissue. Similar hair follicle density and protein fractions in SC are also found (Jung e Maibach, 2015). The porcine skin should not be scalded or flamed after animal sacrificing because this procedure compromises the tissue integrity, affecting drug transport rate (Flaten et al., 2015).

Rodent skin has also been widely used in e*x vivo* permeation studies due to the availability, simple handling and relatively low cost. On the other hand, studies have suggested that this tissue is more permeable than human skin (Barber et al., 1992; Schmook et al., 2001; Van Ravenzwaay e Leibold, 2004), wich may be atributted to anatomical or morphology differences. In rat skin, the most used rodent model, SC and epidermis are thinner, the hair follicle density is higher, intercellular lipid composition of the SC is different and number of corneocyte layers is lower (Capt et al., 2007).

Table 2. Comparison between human and animal skin structure.

Animal model/structural or biochemical chemistry	Human skin	Porcine skin	Rat skin	Mouse skin	Reference
Stratum corneum thickness (μm)	18.2	17.5	15.4	8.8	(Sato et al., 1991)
Epidermis thickness (μm)	51.2	50.7	23.8	18.0	(Sato et al., 1991)
Hair density (per cm^2)	29	22	1,598	5,045	(Mangelsdorf et al., 2014)
Total lipid content in epidermis (% of dry weight)	±10	±8	±20	ND	(Gray e Yardley, 1975)
Distribution of non-polar lipids in epidermis	35.5	30.2	64.2	ND	(Gray e Yardley, 1975)

(% of total lipids)					
Cholesterol content in epidermis (% of total nonpolar lipids)	25	30	18	ND	(Gray e Yardley, 1975)
Ceramide content in epidermis (% of total nonpolar lipids)	3	3	Not detected	ND	(Gray e Yardley, 1975)
SC lipid arrangement	Denser orthorhombic lattice	Hexagonal lattice	ND	ND	(Caussin et al., 2008)

*All parameters were represented by mean values. ND = not determined.

In a study performed by van de Sandt et al. (2004), the permeation of TST and CF in both human and rat tissues were compared. No differences were observed between the mean absorption through human and rat skin for TST. On the other hand, the maximum absorption rate and the total penetration of CF through rat skin were clearly higher than the mean value obtained in human skin (van de Sandt et al., 2004).

A disturbed skin barrier model was also purposed by Schlupp et al. (2014) aiming to obtain more realistic information in situations in that stratum corneum is injured. Cutaneous permeation of CF and TST was evaluated in tape-stripped and abraded skin compared to untreated skin. Porcine skin was selected for the assays. Permeation or retention enhancement was higher for CF than for the TST in the modified skin. TST permeation, for example, increased by 1.9- and 2.1-fold through tape-stripped and abraded skin, respectively. No significant differences in absorption rate was found between the new abrasion method with an aluminum-coated sponge and the tape-stripping traditional method. The *stratum corneum* showed to be less restrictive for lipophilic compounds than for hydrophilic compounds (Schlupp et al., 2014).

Given that rat skin present impaired barrier properties, Netzlaff et al. (2006) included bovine udder skin in a new transport study comparing different animal models. The K_p values of CF through the human, porcine and bovine skin were 0.42, 1.54 and 8.27 ($x10^{-7}$) cm/s, respectively. For TST, these values were 2.31, 1.29 and 5.42 ($x10^{-7}$) cm/s in human, porcine and bovine skin, respectively. Bovine udder skin provided a higher permeation for the tested molecules than human and porcine skin. The tissue constitution was analyzed to make clearer the result understanding. A negative correlation between fatty acid/triglyceride contents in tissues and

permeation rate was found. Bovine udder skin is characterized by a lower content of fatty acids and triglycerides than porcine and human skin, which resulted in higher drug transport. This hypothesis is also supported by the fact that a greater permeation rate was observed for CF than TST. These tissues presented a similar cholesterol composition and epidermal thickness. On the other hand, bovine udder skin presented a much higher density of hair follicles than human skin (about 6/cm^2) and porcine skin (30–36 follicles/cm^2). This characteristic could also contribute to explain the higher permeation rate of CF in bovine udder skin (Netzlaff et al., 2006).

Although human skin represents the gold standard model for permeation studies, this tissue may present limitations during investigation of transfollicular transport of drugs. Interfollicular fibers (collagen and elastin) undergo contraction after skin removal. When the skin is stretched for use in the transport assays, the fibers are also stretched, but the network of fibers close to the hair follicles is not affected by this stretching process. Therefore, hair follicles remain in the contracted state and are only available to a limited level for drug permeation. In contrast, porcine ear skin has the advantage of having an intact skin barrier on the upper face while it is still attached to the cartilage. No tissue contraction occurs during skin removal as in the case of excised human skin. On the other hand, the holes of pig ear hair follicles are approximately three times larger in diameter than those of human hair follicles (Lademann et al., 2010).

In addition to comparing drug permeation in different animal skin models with variation in the follicle density, hair follicle-blocking agents are also useful in mechanistic studies. Porcine skin treated with nail polish was used to investigate the contribution of transfollicular pathway on the transport of CF and TST. Although many studies suggest that transfollicular permeation is preferentially used for highly hydrophilic and high molecular weight molecules (Mitragotri, 2003; Ogiso et al., 2002), the presence of the hair follicle-blocking agent also impacted on the transport rate of TST. In untreated porcine skin, TST presented a K_p value of 4.60 x 10^{-8} cm/s. When hair follicles were individually closed or skin was completely covered, this parameter was reduced to 4.40 and 0.32 x 10^{-8} cm/s, respectively (Stahl et al., 2012). The presence of androgen receptors in follicles could explain the higher affinity of TST for these sites compared to other lipophilic drugs, but further investigations are still needed.

5. SYNTHETIC/ARTIFICIAL MEMBRANES

Although human excised skin is regarded as the gold standard for permeation studies, the relative difficulty of obtaining human skin samples, ethical aspects and the high variability of sources have contributed to the use of artificial membranes (Hadgraft, 2004). In this way, artificial membranes composed of cellulose acetate, dimethylpolysiloxane, hydrocarbons, long-chain alcohols, isopropyl myristate or skin lipids may be considered (Ottaviani et al., 2006) due to the easy availability and result reproducibility (Abd et al., 2016). They are useful to understand the organization of the SC and the impact of each component on the barrier properties (Janůšová et al., 2011; Küchler et al., 2013). Unlike the cellulose acetate membranes, hydrophobic membranes may mimic the drug partitioning into lipids (Jetzer et al., 1986). Hydrophilic membranes, in turn, may be useful as a damaged skin model, where the relatively aqueous dermis is the only remaining barrier (Houk and Guy, 1988).

These membranes may be placed in the interface of the diffusion chambers or prepared directly on the PAMPA (**P**arallel **A**rtificial **M**embrane **P**ermeability **A**ssay) apparatus (Figure 2). This latter assay may be considered for rapid screening of passively transported drugs through skin. An artificial liquid membrane separates the donor and receptor compartments in 96-well plates. The receptor compartment consists of a buffer solution, with or without a solubilizing agent, and the donor compartment contains the drug solution (Neupane et al., 2020).

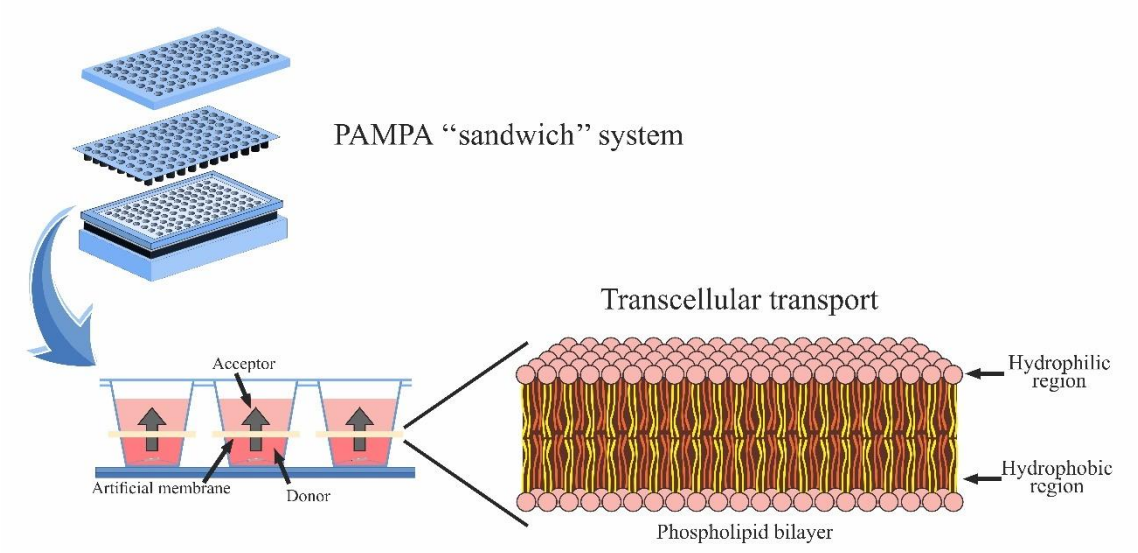


Figure 2. Schematic representation of PAMPA model.

Ottaviani et al. (2006) evaluated the transport rate of CF, TST and other drugs by considering three different membrane compositions (100% silicone; 100% isopropyl myristate-IPM and 70% silicone/30% isopropyl myristate) in PAMPA assay. The liquid silicone membrane allowed a sufficient permeation of TST whereas no permeation was observed for the CF. The high hydrophobicity of this membrane was responsible for these findings. As the SC consists of two alternating lipophilic and hydrophilic layers, IMP was immobilized on hydrophobic PVDF filters to evaluate the transport rate of drugs. Although this membrane type has improved permeation rate of CF and TST, low correlation with data in human skin was found (conclusion based on all tested molecules). Moreover, the differences in K_p values of molecules with different log P values were reduced, i.e., assay showed to be less discriminatory. Finally, the authors combined both silicone and IMP in a same membrane, achieving higher correlation with data in human skin. IMP would play a role as a H-bond acceptor in the membrane, similarly to proteins of stratum corneum (Ottaviani et al., 2006).

Skin constituents such as cholesterol, free fatty acids, and the ceramides analogues (namely synthetic certramides, long-chain tartaric acid diamides) were also selected to prepare artificial membranes for PAMPA assay. These new certramides are structurally similar to the natural ceramides with comparable molar mass, H-bond donor/acceptors and high lipophilicity. CF, TST and other 20 molecules were considered as drug models for transport studies. A higher correlation between permeation rate of TST in PAMPA assay and human skin was found when compared to CF. The effect of PEG 400, a known solubilizing agent and chemical penetration enhancer, on the permeation rate of these molecules was also evaluated. Interestingly, an increased transport rate in presence of this agent was found. PEG 400 would be able to dissolve membrane constituents, reducing the barrier properties (Sinkó et al., 2012). This finding limits the application of PAMPA-skin assay for mechanistic studies with new chemical penetration enhancer or formulations.

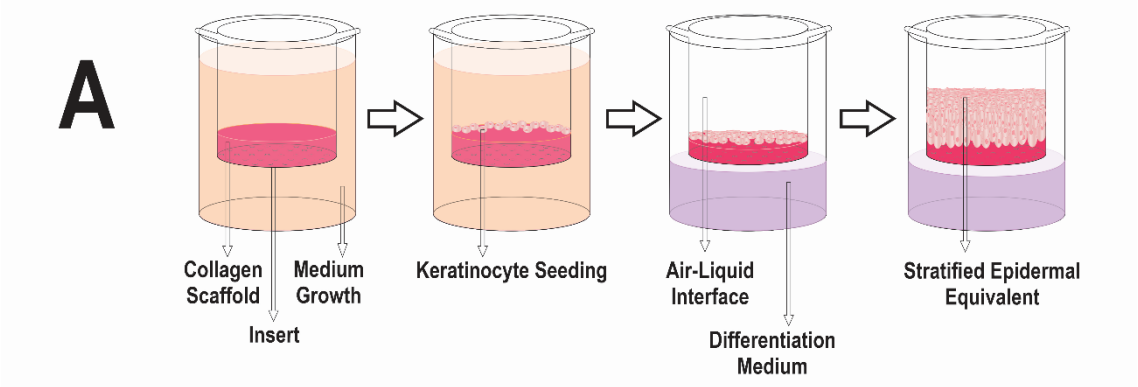
The permeation of drugs through artificial membranes (isopropyl myristate, certramides and Strat-M™) was also compared to that of porcine skin. Strat-M™ are commercial membranes composed of multiple layers of polyether sulfone that result in a morphology similar to human skin with a very tight surface layer. In

addition to CF and TST, other 4 compounds were selected for permeation studies. Saturated and unsaturated concentrations of these compounds as well as different vehicles were tested. A stronger correlation between membrane and diffusion cell results was obtained when only saturated concentrations were considered (r^2 = 0.60, 0.63 and 0.66 for the Strat-M™, certramide and isopropyl myristate membranes, respectively). The stronger *in vivo-ex vivo* correlation found for isopropyl myristate membranes may be explained for their amphiphilic nature (SC presents both polar and nonpolar sites, which are represented by proteins and lipids, respectively). Therefore, the authors suggest that artificial membranes in PAMPA apparatus could be useful in an initial screening of compounds (Karadzovska e Riviere, 2013).

In summary, synthetic membranes could be considered as skin models when (1) the stratum corneum represents the major barrier resistance to transport; (2) the active compound is metabolically inert and not specifically bound in viable skin (this is not the case of TST, which is able to interact with androgen receptors in skin); (3) formulations without chemical penetration enhancers able to interact with the membrane are tested; and (4) *in vivo* assays of similar design can be easily performed for result validation.

6. RECONSTRUCTED SKIN MODELS

Reconstructed human skin models were developed in the 1980s to replace animal experimentation (Nakamura et al., 2018). In these skin models, layers of human cells are usually grown on a polymer matrix (e.g., collagen scaffolds) (Figure 3). Different cell types may be considered to achieve a structure of the desired composition and complexity. Reconstructed skin models may be designed to simulate only the epidermis or full-thickness skin (Flaten et al., 2015; Küchler et al., 2013).



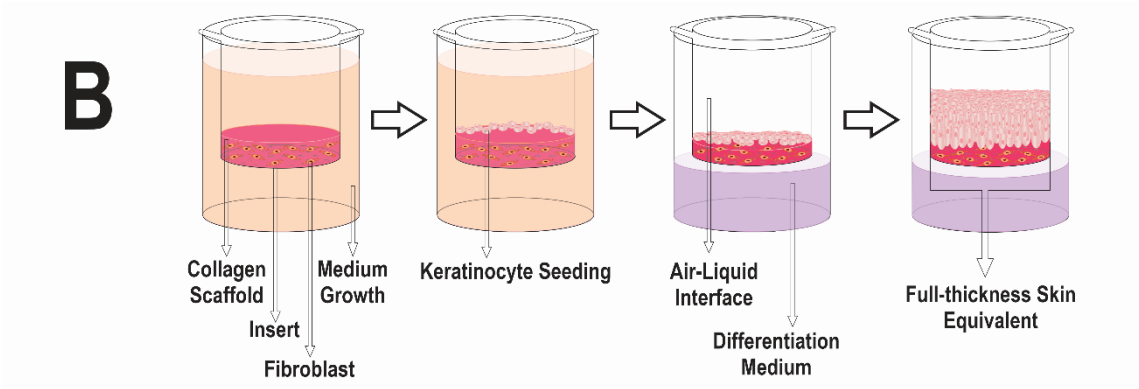


Figure 3. Reconstructed human skin models for drug transport studies. In the model A (stratified epidermal equivalent), keratinocytes are seeded onto a collagen scaffold. In the model B (full-thickness skin equivalent), the collagen scaffold is previously populated with fibroblasts. The air-liquid interface is crucial for differentiation of keratinocyte in both models.

The commercial systems SkinEthic® (SkinEthics, France), epiCS® (CellSystems, Germany), and EpiDerm® (MatTek Corporation, USA) consist of epidermal keratinocytes cultured on polycarbonate membrane. EpiSkin® (L'Oréal, France), in turn, is composed of stratified differentiated human keratinocytes cultured on a dermal matrix (type I bovine collagen matrix with type IV human collagen film). In advanced skin models such as Phenion® (Henkel, Germany) and NeoDerm® (Tegoscience, Korea), human fibroblasts are initially cultivated on a collagen matrix, followed by deposition of an epidermal layer composed of human keratinocytes (Yun et al., 2018).

These skin models exhibit metabolic activity, an interesting advantage compared to the synthetic membranes (Küchler et al., 2013). Therefore, drug transport and metabolism may be evaluated with the same model. Interestingly, the esterase activity in reconstructed skin models has been correlated to that of native human skin (Gysler et al., 1999). Similar polar and nonpolar metabolites of TST were also found in a comparative study between a reconstructed skin model and native skin (Slivka, 1992). Ernesti et al. (1992) also found a similar metabolic profile when skin constructs composed of fibroblasts, keratinocytes and collagen were compared human skin. The percentages of TST metabolized to 5α-DHT and

androsterone was similar among the reconstructed skin model and leg skin (approximately 15%).

Although these models have already been approved by OECD for skin corrosivity (OECD, 2004a) and phototoxicity assays (OECD, 2004b), their application in drug absorption studies require previous validation with reference skin models (OECD, 2004c). A series of comparative studies has been performed in the last two decades considering this aspect (Table 3). Schreiber et al. (2005), for example, compared the cutaneous permeation of CF and TST through reconstructed epidermis models (SkinEthic® and EpiDerm®) with porcine and human skin, which serve as a reference. For both molecules, SkinEthic® was more permeable than EpiDerm®, followed by porcine skin and then human epidermis. K_p values of CF through SkinEthic®, EpiDerm®, porcine skin and human epidermis were 777.6, 176.4, 31.7 and 7.6 x (10^{-5}) cm/h, respectively. For TST, the same rank through different membranes was obtained, which was 212.4, 122.4, 11.5 and 9.4 x (10^{-4}) cm/h, respectively. Less differences in the permeation rate between reconstructed epidermis and reference skin models were found for TST than CF. The lag time obtained from the reconstructed epidermis models was similar to that of human epidermis. The authors explained this finding based on thermal treatment of human tissues. Taken together, these results showed than reconstructed epidermis models were more permeable than reference models (Schreiber et al., 2005).

Schäfer-Korting et al. (2006) performed a similar comparative study, including EpiSkin® and bovine udder skin in this new analysis. A similar permeation of TST through the EpiDerm® and EpiSkin® model was found (K_p = 2.89 versus 2.11 x 10^{-6} cm/s, respectively), while the SkinEthic® model showed to be more permeable (K_p = 6 x 10^{-6} cm/s). The K_p values of CF through the human epidermis, porcine skin and bovine udder skin were 0.42, 0.08 and 0.32 x (10^{-6}) cm/s, respectively. For the CF, EpiSkin® model showed to be less permeable, followed by EpiSkin® and SkinEthic® (K_p = 0.24, 2.77 and 3.63 x 10^{-6} cm/s). The K_p values of TST through human epidermis, pig skin and bovine udder skin were 0.06, 0.07 and 0.63 x (10^{-6}) cm/s, respectively. Overall, reconstructed epidermis models demonstrated a higher K_p and shorter lag time than animal and human tissues for both drugs. Lower free fatty acid and ceramide fractions or low expression of cytokines and growth factors associated to hyper-proliferation of the

epidermal cells could lead to an impaired barrier, explaining these findings (Ponec et al., 2002). Inter-laboratory variation was also evaluated (*n*=5). Low K_p variation was found (9.3% for CF and almost zero for TST), suggesting that the standardized protocols could be efficiently transferred (Schäfer-Korting et al., 2006).

In a new study performed by the same research team involving ten different laboratories, CF, TST and seven other compounds presenting different physicochemical properties were tested in these skin models. Overall, the K_p values were close to that of previous study. For TST, the SkinEthic® showed to be more permeable than the EpiDerm® and EpiSkin®. For the CF, SkinEthic® and EpiSkin® were more permeable. For both drugs, reconstructed epidermis models demonstrated higher K_p values than human and animal tissues. The K_p obtained for CF with human epidermis and pig skin model were higher than in the previous study. The authors attributed these differences to tissue preparation/origin. In summary, the permeation ranking of drugs was similar through all membranes; however, the reconstructed epidermis models showed higher permeation rate than human epidermis and pig skin. As already reported, reconstructed epidermis models also showed more reproducible results (Schäfer-Korting et al., 2008).

Drug transport studies have also been performed in full-thickness skin models (dermis and epidermis). In this study, the permeation of CF and TST through Phenion® full-thickness skin model was compared to that of porcine skin. As expected, Kp values of TST in Phenion® model were higher than porcine skin (1.58 vs. 0.08 x 10^{-6} cm/s, respectively). For CF, this difference was even greater (6.7/6.73 vs. 0.03 x 10^{-6} cm/s, respectively). When compared to EpiDerm, SkinEthic and EpiSkin, this model had a slightly weaker barrier for CF transport whereas it would retard the permeation of lipophilic compounds such as TST. In the lag time analysis, lower values were obtained with Phenion® model, which had already been observed with other reconstructed skin models. For TST, Phenion® model (0.56 h) resulted in lag time values even shorter than EpiSkin®, which was associated with structural differences. In Phenion® model, fibroblasts are embedded into loosely packed newly synthesized extracellular matrix fibers whereas keratinocytes are grown on a densely packed collagen layer in EpiSkin® (Ackermann et al., 2010).

All permeation studies mentioned in this review demonstrated that reconstructed skin models result in higher drug permeation rate and shorter lag time of CF and TST than reference models (human skin or porcine skin). This impaired barrier property could be justified by differences in lipid composition compared to native skin (Figure 4). The major skin barrier lipids are also produced *in vitro*; however, in different proportions than those found in native skin (Ponec et al., 2002). Incomplete ceramide profiles and reduced content of fatty acids are found in the EpidDerm®, SkinEthic® and EpiSkin models®. In fact, the content of free fatty acids is very low in EpiDerm® and Episkin® models (approximately 2%) and moderate in SkinEthic cultures (approximately 6%) as compared with native epidermis (10%). Reconstructed skin models present low levels of ceramides 5 and 6 and the absence of ceramide 7 (more polar ceramides). The SkinEthic® model, which presented higher transport rate for both drugs, is characterized by a lower total lipid content than other reconstructed skin models (0.29 vs. 0.438 and 0.526 mg/cm^2 in EpiSkin® and EpiDerm®, respectively) (Ponec et al., 2000). Consequently, this skin model would present weaker barrier properties. Another hypothesis for explaining these differences between reconstructed skin models and native skin is the lipid organization in stratum corneum. In native SC lipids, the lateral packing is predominantly orthorhombic while a hexagonal packing is found in reconstructed SC (Ponec et al., 2001).

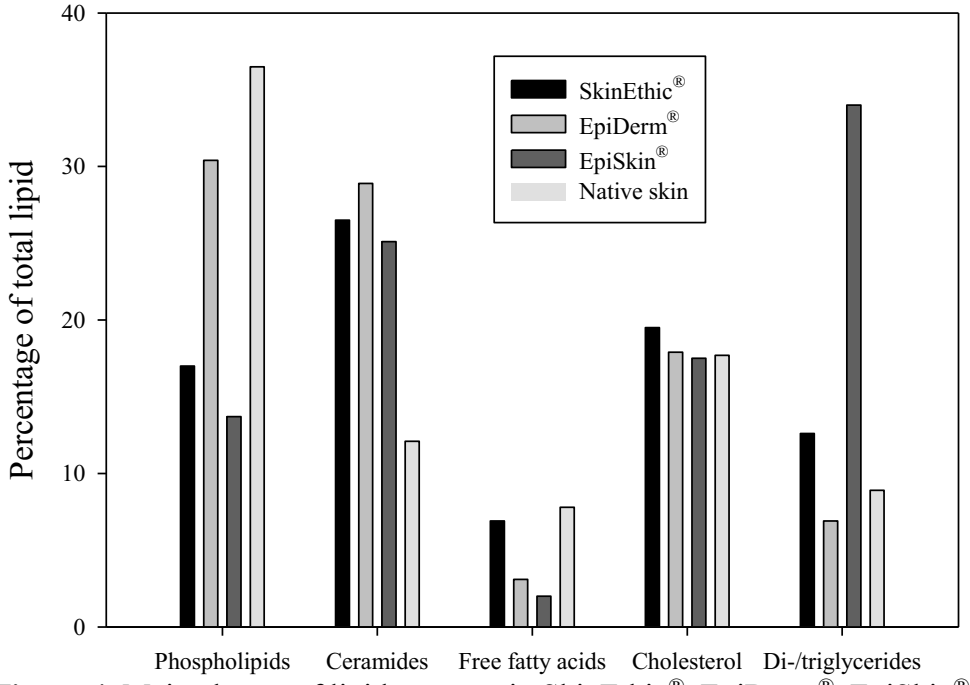


Figure 4. Main classes of lipids present in SkinEthic®, EpiDerm®, EpiSkin® compared to native skin. Data were obtained from Ponec et al. (2002).

The role of the dermis in drug transport through reconstructed skin models is still not well defined as few studies with advanced skin models have been performed to date. In a study with the Phenion® model, the permeation of TST was more retarded. This finding was explained as it does not present subcutaneous vasculatures and its environment is more aqueous than native skin (Van Gele et al., 2011).

Another point worth to mention is that these models are not appropriate to study the appendageal transport of molecules. The absence of hair follicles or sweat glands target the drugs to inter- and transcellular pathways (Neupane et al., 2020). Skin appendages such as hair follicles and sebaceous glands have been considered an important transport route of hydrophilic molecules as CF (Otberg et al., 2008) and for steroids as TST (Hueber et al., 1994). This fact could also explain the differences in transport rate of CF and TST between reconstructed skin models and native skin.

Table 3. Permeation parameters (permeation coefficient and lag time) for caffeine and testosterone obtained from reconstructed skin models and reference tissues.

Model skin	Permeation coefficient values (cm/s, $\times 10^{-6}$)	Lag time (h)	Reference
Testosterone			
Human epidermis (reference model)	0.26	0.03	(Schreiber et al., 2005)
Porcine skin (reference model)	0.32	7.63	
EpiDerm®	3.40	0.00	
SkinEthic®	5.90	0.01	
Human epidermis (reference model)	0.42	1.03	(Schäfer-Korting et al., 2006)
Porcine skin (reference model)	0.08	-0.13	
Bovine udder skin (reference model)	0.32	1.19	
EpiDerm®	2.89	0.00	
SkinEthic®	6.00	0.14	
EpiSkin®	2.11	0.93	
Human epidermis (reference model)	0.50	0.51	(Schäfer-Korting et al., 2008)
Porcine skin (reference model)	0.25	6.66	
EpiDerm®	2.78	0.08	
SkinEthic®	5.70	0.07	
EpiSkin®	2.10	0.52	
Porcine skin	0.08	5.60	(Ackermann et al.,

(reference model)			2010)
Phenion®	1.58	0.56	
Caffeine			
Human epidermis (reference model)	0.02	0.00	(Schreiber et al., 2005)
Porcine skin (reference model)	0.08	10.7	
EpiDerm®	0.49	0.16	
SkinEthic®	2.16	0.00	
Human epidermis (reference model)	0.06	1.73	(Schäfer-Korting et al., 2006)
Porcine skin (reference model)	0.07	3.92	
Bovine udder skin	0.63	1.88	
EpiDerm®	0.24	0.33	
SkinEthic®	3.63	0.14	
EpiSkin®	2.77	1.04	
Human epidermis (reference model)	0.28	1.75	(Schäfer-Korting et al., 2008)
Porcine skin (reference model)	0.31	6.92	
EpiDerm®	0.59	0.35	
SkinEthic®	4.32	0.17	
EpiSkin®	2.94	0.81	
Porcine skin (reference model)	0.03	6.73	(Ackermann et al., 2010)
Phenion® (1)	6.70	0.55	
Phenion® (2)	6.73	0.43	

Permeation coefficient values obtained by Schreiber et al. (2005) were converted in "cm/s" to make the comparison clearer.

Efforts to improve reconstructed skin models have been constant. Zimoch et al. (2021) have recently reported that the presence of hypodermis in these skin models is crucial for keratinocyte maturation (Zimoch et al., 2021). We could hypothesize that the presence of adipocytes would contribute to improve the barrier properties of the stratum corneum; however, this additional layer could retard the amount of compound permeated. Transport assays in this skin model have not yet been considered.

Intra-specie variation or origin of cells used in reconstructed skin models, in turn, also seems to impact the state of cell differentiation and barrier properties. Girardeau-Hubert et al. (2019), for example, evaluated differences in reconstructed skin models with human keratinocytes and papillary fibroblasts (full-thickness skin model) when epidermal cells from African and Caucasian subjects

were obtained. The terminal differentiation of Caucasian keratinocytes was improved in relation to African keratinocytes. Caucasian epidermis presented an up-regulated signaling for ceramide metabolism and an up-regulated set of genes associated with late epidermal differentiation in contrast to African epidermis, which can be mainly related to proflaggrin synthesis and processing. Therefore, differences in epidermal morphogenesis and differentiation between keratinocytes of Caucasian and African skin types could result in variable skin behaviors (e.g., permeation rate).

7. FINAL CONSIDERATIONS

Although reconstructed skin models have been shown to be effective in predicting the *in vitro* cytotoxicity of compounds, they result in an increased transport rate compared to human skin. Impaired barrier properties would contribute to explain these differences. In addition, these skin models predict only part of the absorption mechanisms, not including transfollicular transport. On the other hand, they are more advantageous than synthetic membranes and animal tissues in predicting metabolic processes due to expression of skin enzymes. In synthetic membranes, no enzyme activity is present. In animal models, tissue preparation is time consuming and may involve heat treatment, which affects the level of enzymatic activity in an uncontrolled way. The relationship between freezing time or temperature and enzyme level is still not very well known, which requires further investigations.

Efforts have been performed to achieve the same lipid composition among reconstructed and physiological skin; however, differences in lipid organization are still observed. Vehicles included in transdermal formulations may also impact on skin barrier integrity. Solvents such as ethanol or surfactants, which are often used to achieve the “sink” condition in permeation assays with lipophilic drugs, have their use limited in reconstructed skin models. These agents can solubilize membrane lipids, affecting the barrier integrity. Several research groups have tried to study as culture conditions affect the cell phenotype and barrier properties, but the development of hair follicles and blood vessels in these models is still a long way off. On the other hand, this learning will allow a faster development of unhealthy/injured skin models.

Human and animal tissues still remain as the most representative skin models for drug absorption studies, particularly when new molecules are investigated or for mechanistic purposes (e.g., study the transport route of the compound through the skin, action of absorption chemical enhancers). Among the animal models, porcine skin should be prioritized due to a similar composition and permeability to human skin. Synthetic skin models would be useful for comparative tests between different formulations containing molecules where transcellular transport is predominant.

ACKNOWLEDGMENTS

We thank Rafaela E. B. Speroni for her help in preparing the figures.

FUNDING STATEMENT

No funding for the publication of this article was received by the authors.

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.

REFERENCES

Abd, E., Yousef, S.A., Pastore, M.N., Telaprolu, K., Mohammed, Y.H., Namjoshi, S., Grice, J.E., Roberts, M.S., 2016. Skin models for the testing of transdermal drugs. Clin. Pharmacol. Adv. Appl. 8, 163–176. https://doi.org/10.2147/CPAA.S64788

Ackermann, K., Lombardi Borgia, S., Korting, H.C., Mewes, K.R., Schäfer-Korting, M., 2010. The phenion® full-thickness skin model for percutaneous absorption testing. Skin Pharmacol. Physiol. 23, 105–112. https://doi.org/10.1159/000265681

Atrux-Tallau, N., Pirot, F., Falson, F., Roberts, M.S., Maibach, H.I., 2007. Qualitative and quantitative comparison of heat separated epidermis and dermatomed skin in percutaneous absorption studies. Arch. Dermatol. Res. 299, 507–511. https://doi.org/10.1007/s00403-007-0789-y

Barber, E.D., Teetsel, N.M., Kolberg, K.F., Guest, D., 1992. A comparative study of the rates of in vitro percutaneous absorption of eight chemicals using rat and human skin. Fundam. Appl. Toxicol. 19, 493–497. https://doi.org/10.1016/0272-0590(92)90086-W

Barbero, A.M., Frasch, H.F., 2009. Pig and guinea pig skin as surrogates for human in vitro penetration studies: A quantitative review. Toxicol. Vitr. 23, 1–13. https://doi.org/10.1016/j.tiv.2008.10.008

Bouwstra, J., 2003. Structure of the skin barrier and its modulation by vesicular formulations. Prog. Lipid Res. 42, 1–36. https://doi.org/10.1016/S0163-7827(02)00028-0

Brain, K., Walters, K., Watkinson, A., 2002. Methods for Studying Percutaneous Absorption, Drugs and the Pharmaceutical Sciences, in: Percutaneous Absorption Drugs, Cosmetics, Mechanisms, Methods. p. 197–269.

Capt, A., Luzy, A.P., Esdaile, D., Blanck, O., 2007. Comparison of the human skin grafted onto nude mouse model with in vivo and in vitro models in the prediction of percutaneous penetration of three lipophilic pesticides. Regul. Toxicol. Pharmacol. 47, 274–287. https://doi.org/10.1016/j.yrtph.2006.11.008

Caussin, J., Gooris, G.S., Janssens, M., Bouwstra, J.A., 2008. Lipid organization in

human and porcine stratum corneum differs widely, while lipid mixtures with porcine ceramides model human stratum corneum lipid organization very closely. Biochim. Biophys. Acta - Biomembr. 1778, 1472–1482. https://doi.org/10.1016/j.bbamem.2008.03.003

Cross, S.E., Magnusson, B.M., Winckle, G., Anissimov, Y., Roberts, M.S., 2003. Determination of the effect of lipophilicity on the in vitro permeability and tissue reservoir characteristics of topically applied solutes in human skin layers. J. Invest. Dermatol. 120, 759–764. https://doi.org/10.1046/j.1523-1747.2003.12131.x

Dominguez, S., Mackert, G.A., Dobke, M.., 2017. Nanotechnology to enhance transdermal delivery of hydrophilic humectants for improved skin care: a model for therapeutic applications, in: Andronescu, E., Grumezescu, A.M. (Orgs.), Nanostructures for Drug Delivery. Elsevier, p. 919–939.

Ernesti, A.M., Swiderek, M., Gay, R., 1992. Absorption and metabolism of topically applied testosterone in an organotypic skin culture. Ski. Pharmacol. 5, 146–153. https://doi.org/10.1159/000211031

Esposto Biondo, N., Fretes Argenta, D., Schneider Rauber, G., Caon, T., 2021. How to define the experimental conditions of skin permeation assays for drugs presenting biopharmaceutical limitations? The experience with testosterone. Int. J. Pharm. 607, 120987. https://doi.org/10.1016/j.ijpharm.2021.120987

Évora, A.S., Adams, M.J., Johnson, S.A., Zhang, Z., 2021. Corneocytes: Relationship between Structural and Biomechanical Properties. Skin Pharmacol. Physiol. 34, 146–161. https://doi.org/10.1159/000513054

Flaten, G.E., Palac, Z., Engesland, A., Filipović-Grčić, J., Vanić, Ž., Škalko-Basnet, N., 2015. In vitro skin models as a tool in optimization of drug formulation. Eur. J. Pharm. Sci. 75, 10–24. https://doi.org/10.1016/j.ejps.2015.02.018

Girardeau-Hubert, S., Deneuville, C., Pageon, H., Abed, K., Tacheau, C., Cavusoglu, N., Donovan, M., Bernard, D., Asselineau, D., 2019. Reconstructed Skin Models Revealed Unexpected Differences in Epidermal African and Caucasian Skin. Sci. Rep. 9, 7456. https://doi.org/10.1038/s41598-019-43128-3

Gray, G.M., Yardley, H.J., 1975. Lipid compositions of cells isolated from pig, human,

and rat epidermis. J. Lipid Res. 16, 434–40.

Gysler, A., Kleuser, B., Sippl, W., Lange, K., Korting, H.C., Höltje, H.D., Korting, H.C., 1999. Skin penetration and metabolism of topical glucocorticoids in reconstructed epidermis and in excised human skin. Pharm. Res. 16, 1386–91. https://doi.org/10.1023/a:1018946924585

Hadgraft, J., 2004. Skin deep. Eur. J. Pharm. Biopharm. 58, 291–299. https://doi.org/10.1016/j.ejpb.2004.03.002

Haq, A., Dorrani, M., Goodyear, B., Joshi, V., Michniak-Kohn, B., 2018. Membrane properties for permeability testing: Skin versus synthetic membranes. Int. J. Pharm. 539, 58–64. https://doi.org/10.1016/j.ijpharm.2018.01.029

Houk, J., Guy, R.H., 1988. Membrane models for skin penetration studies. Chem. Rev. 88, 455–472. https://doi.org/10.1021/cr00085a001

Hueber, F., Schaefer, H., Wepierre, J., 1994. Role of transepidermal and transfollicular routes in percutaneous absorption of steroids: In vitro studies on human skin. Skin Pharmacol. Physiol. 7, 237–244. https://doi.org/10.1159/000211300

Im, J.E., Kim, H.Y., Lee, J.D., Park, J.J., Kang, K.S., Kim, K.B., 2021. Effect of application amounts on in vitro dermal absorption test using caffeine and testosterone. Pharmaceutics 13. https://doi.org/10.3390/pharmaceutics13050641

Janůšová, B., Zbytovská, J., Lorenc, P., Vavrysová, H., Palát, K., Hrabálek, A., Vávrová, K., 2011. Effect of ceramide acyl chain length on skin permeability and thermotropic phase behavior of model stratum corneum lipid membranes. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1811, 129–137. https://doi.org/10.1016/j.bbalip.2010.12.003

Jetzer, W.E., Huq, A.S., Ho, N.F.H., Flynn, G.L., Duraiswamy, N., Condie, L., 1986. Permeation of Mouse Skin and Silicone Rubber Membranes by Phenols: Relationship to In Vitro Partitioning. J. Pharm. Sci. 75, 1098–1103. https://doi.org/10.1002/jps.2600751116

Jung, E.C., Maibach, H.I., 2015. Animal models for percutaneous absorption. J. Appl. Toxicol. 35, 1–10. https://doi.org/10.1002/jat.3004

Karadzovska, D., Riviere, J.E., 2013. Assessing vehicle effects on skin absorption using

artificial membrane assays. Eur. J. Pharm. Sci. 50, 569–576. https://doi.org/10.1016/j.ejps.2013.02.020

Küchler, S., Strüver, K., Friess, W., 2013. Reconstructed skin models as emerging tools for drug absorption studies. Expert Opin. Drug Metab. Toxicol. 9, 1255–1263. https://doi.org/10.1517/17425255.2013.816284

Lademann, J., Richter, H., Meinke, M., Sterry, W., Patzelt, A., 2010. Which Skin Model Is the Most Appropriate for the Investigation of Topically Applied Substances into the Hair Follicles? Skin Pharmacol. Physiol. 23, 47–52. https://doi.org/10.1159/000257263

Mangelsdorf, S., Vergou, T., Sterry, W., Lademann, J., Patzelt, A., 2014. Comparative study of hair follicle morphology in eight mammalian species and humans. Ski. Res. Technol. 20, 147–154. https://doi.org/10.1111/srt.12098

Mitragotri, S., 2003. Modeling skin permeability to hydrophilic and hydrophobic solutes based on four permeation pathways. J. Control. Release 86, 69–92. https://doi.org/10.1016/S0168-3659(02)00321-8

Moser, K., Kriwet, K., Naik, A., Kalia, Y.N., Guy, R.H., 2001. Passive skin penetration enhancement and its quantification in vitro. Eur. J. Pharm. Biopharm. 52, 103–112. https://doi.org/10.1016/S0939-6411(01)00166-7

Nakamura, M., Haarmann-Stemmann, T., Krutmann, J., Morita, A., 2018. Alternative test models for skin ageing research. Exp. Dermatol. 27, 495–500. https://doi.org/10.1111/exd.13519

Netzlaff, F., Schaefer, U.F., Lehr, C.-M., Meiers, P., Stahl, J., Kietzmann, M., Niedorf, F., 2006. Comparison of bovine udder skin with human and porcine skin in percutaneous permeation experiments. ATLA Altern. to Lab. Anim. 34, 499–513.

Neupane, R., Boddu, S.H.S., Renukuntla, J., Babu, R.J., Tiwari, A.K., 2020. Alternatives to Biological Skin in Permeation Studies: Current Trends and Possibilities. Pharmaceutics 12, 152. https://doi.org/10.3390/pharmaceutics12020152

OECD, 2004a. OECD guideline for the testing of chemicals, No. 431., in: OECD (Org.), In vitro skin corrosion: human skin model test. OECD, Paris, France, p. 8.

OECD, 2004b. OECD guideline for the testing of chemicals, No. 432., in: OECD (Org.), In vitro 3T3 NRU phototoxicity test. OECD, Paris, France, p. 15.

OECD, 2004c. Guidance Document for the Conduct of Skin Absorption Studies. OECD Series on Testing and Assessment. https://doi.org/10.1787/9789264078796-en

OECD, 2000a. Guideline for the testing of chemicals. Skin absorption: in vivo method.

OECD, 2000b. Draft guidance document for the conduct of skin absorption studies. Environ. Heal. Saf. Publ.

OECD, 2000c. Test Guidelines Program. Percutaneous absorption testing: is there a way to consensus?

Ogiso, T., Shiraki, T., Okajima, K., Tanino, T., Iwaki, M., Wada, T., 2002. Transfollicular Drug Delivery: Penetration of Drugs Through Human Scalp Skin and Comparison of Penetration Between Scalp and Abdominal Skins In Vitro. J. Drug Target. 10, 369–378. https://doi.org/10.1080/1061186021000001814

Otberg, N., Patzelt, A., Rasulev, U., Hagemeister, T., Linscheid, M., Sinkgraven, R., Sterry, W., Lademann, J., 2008. The role of hair follicles in the percutaneous absorption of caffeine. Br. J. Clin. Pharmacol. 65, 488–492. https://doi.org/10.1111/j.1365-2125.2007.03065.x

Ottaviani, G., Martel, S., Carrupt, P.-A., 2006. Parallel artificial membrane permeability assay: A new membrane for the fast prediction of passive human skin permeability. J. Med. Chem. 49, 3948–3954. https://doi.org/10.1021/jm060230+

Planz, V., Lehr, C.M., Windbergs, M., 2016. In vitro models for evaluating safety and efficacy of novel technologies for skin drug delivery. J. Control. Release 242, 89–104. https://doi.org/10.1016/j.jconrel.2016.09.002

Ponec, M., Boelsma, E., Gibbs, S., Mommaas, M., 2002. Characterization of Reconstructed Skin Models. Skin Pharmacol. Physiol. 15, 4–17. https://doi.org/10.1159/000066682

Ponec, M., Boelsma, E., Weerheim, A., Mulder, A., Bouwstra, J., Mommaas, M., 2000. Lipid and ultrastructural characterization of reconstructed skin models. Int. J. Pharm. 203, 211–225. https://doi.org/10.1016/S0378-5173(00)00459-2

Ponec, M., Gibbs, S., Pilgram, G., Boelsma, E., Koerten, H., Bouwstra, J., Mommaas, M., 2001. Barrier Function in Reconstructed Epidermis and Its Resemblance to Native Human Skin. Skin Pharmacol. Physiol. 14, 63–71. https://doi.org/10.1159/000056392

Prausnitz, M.R., Langer, R., 2008. Transdermal drug delivery. Nat. Biotechnol. 26, 1261–1268. https://doi.org/10.1038/nbt.1504

Raney, S.G., Franz, T.J., Lehman, P.A., Lionberger, R., Chen, M.-L., 2015. Pharmacokinetics-Based Approaches for Bioequivalence Evaluation of Topical Dermatological Drug Products. Clin. Pharmacokinet. 54, 1095–1106. https://doi.org/10.1007/s40262-015-0292-0

Roberts, M.S., Cheruvu, H.S., Mangion, S.E., Alinaghi, A., Benson, H.A.E., Mohammed, Y., Holmes, A., van der Hoek, J., Pastore, M., Grice, J.E., 2021. Topical drug delivery: History, percutaneous absorption, and product development. Adv. Drug Deliv. Rev. 177, 113929. https://doi.org/10.1016/j.addr.2021.113929

Sato, K., Sugibayashi, K., Morimoto, Y., 1991. Species Differences in Percutaneous Absorption of Nicorandil. J. Pharm. Sci. 80, 104–107. https://doi.org/10.1002/jps.2600800203

Schäfer-Korting, M., Bock, U., Diembeck, W., Düsing, H.-J., Gamer, A., Haltner-Ukomadu, E., Hoffmann, C., Kaca, M., Kamp, H., Kersen, S., Kietzmann, M., Korting, H.C., Krächter, H.-U., Lehr, C.-M., Liebsch, M., Mehling, A., Müller-Goymann, C., Netzlaff, F., Niedorf, F., Rübbelke, M.K., Schäfer, U., Schmidt, E., Schreiber, S., Spielmann, H., Vuia, A., Weimer, M., 2008. The Use of Reconstructed Human Epidermis for Skin Absorption Testing: Results of the Validation Study. Altern. to Lab. Anim. 36, 161–187. https://doi.org/10.1177/026119290803600207

Schäfer-Korting, M., Bock, U., Gamer, A., Haberland, A., Haltner-Ukomadu, E., Kaca, M., Kamp, H., Kietzmann, M., Korting, H.C., Krächter, H.-U., Lehr, C.-M., Liebsch, M., Mehling, A., Netzlaff, F., Niedorf, F., Rübbelke, M.K., Schäfer, U., Schmidt, E., Schreiber, S., Schröder, K.-R., Spielmann, H., Vuia, A., 2006. Reconstructed human epidermis for skin absorption testing: Results of the German prevalidation study. ATLA Altern. to Lab. Anim. 34, 283–294.

Schlupp, P., Weber, M., Schmidts, T., Geiger, K., Runkel, F., 2014. Development and validation of an alternative disturbed skin model by mechanical abrasion to study drug penetration. Results Pharma Sci. 4, 26–33. https://doi.org/10.1016/j.rinphs.2014.09.002

Schmook, F.P., Meingassner, J.G., Billich, A., 2001. Comparison of human skin or epidermis models with human and animal skin in in-vitro percutaneous absorption. Int. J. Pharm. 215, 51–56. https://doi.org/10.1016/S0378-5173(00)00665-7

Schreiber, S., Mahmoud, A., Vuia, A., Rübbelke, M.K., Schmidt, E., Schaller, M., Kandárová, H., Haberland, A., Schäfer, U.F., Bock, U., Korting, H.C., Liebsch, M., Schäfer-Korting, M., 2005. Reconstructed epidermis versus human and animal skin in skin absorption studies. Toxicol. Vitr. 19, 813–822. https://doi.org/10.1016/j.tiv.2005.04.004

Shin, J.-W., Kwon, S.-H., Choi, J.-Y., Na, J.-I., Huh, C.-H., Choi, H.-R., Park, K.-C., 2019. Molecular Mechanisms of Dermal Aging and Antiaging Approaches. Int. J. Mol. Sci. 20, 2126. https://doi.org/10.3390/ijms20092126

Simon, G.A., Maibach, H.I., 2000. The Pig as an Experimental Animal Model of Percutaneous Permeation in Man: Qualitative and Quantitative Observations – An Overview. Skin Pharmacol. Physiol. 13, 229–234. https://doi.org/10.1159/000029928

Sinkó, B., Garrigues, T.M., Balogh, G.T., Nagy, Z.K., Tsinman, O., Avdeef, A., Takács-Novák, K., 2012. Skin-PAMPA: A new method for fast prediction of skin penetration. Eur. J. Pharm. Sci. 45, 698–707. https://doi.org/10.1016/j.ejps.2012.01.011

Sjövall, P., Skedung, L., Gregoire, S., Biganska, O., Clément, F., Luengo, G.S., 2018. Imaging the distribution of skin lipids and topically applied compounds in human skin using mass spectrometry. Sci. Rep. 8, 16683. https://doi.org/10.1038/s41598-018-34286-x

Slivka, S.R., 1992. Testosterone metabolism in an in vitro skin model. Cell Biol. Toxicol. 8, 267–276. https://doi.org/10.1007/BF00156735

Stahl, J., Niedorf, F., Wohlert, M., Kietzmann, M., 2012. The in vitro use of the hair

follicle closure technique to study the follicular and percutaneous permeation of topically applied drugs. ATLA Altern. to Lab. Anim. 40, 51–57.

Supe, S., Takudage, P., 2021. Methods for evaluating penetration of drug into the skin: A review. Ski. Res. Technol. 27, 299–308. https://doi.org/10.1111/srt.12968

van de Sandt, J.J.M., van Burgsteden, J.A., Cage, S., Carmichael, P.L., Dick, I., Kenyon, S., Korinth, G., Larese, F., Limasset, J.C., Maas, W.J.M., Montomoli, L., Nielsen, J.B., Payan, J.-P., Robinson, E., Sartorelli, P., Schaller, K.H., Wilkinson, S.C., Williams, F.M., 2004. In vitro predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study. Regul. Toxicol. Pharmacol. 39, 271–281. https://doi.org/10.1016/j.yrtph.2004.02.004

Van Gele, M., Geusens, B., Brochez, L., Speeckaert, R., Lambert, J., 2011. Three-dimensional skin models as tools for transdermal drug delivery: Challenges and limitations. Expert Opin. Drug Deliv. 8, 705–720. https://doi.org/10.1517/17425247.2011.568937

Van Ravenzwaay, B., Leibold, E., 2004. A comparison between in vitro rat and human and in vivo rat skin absorption studies. Hum. Exp. Toxicol. 23, 421–430. https://doi.org/10.1191/0960327104ht471oa

Verma, A., Jain, S.K., Jain, A., Hurkat, P., 2016. Transfollicular drug delivery: current perspectives. Res. Reports Transdermal Drug Deliv. 1. https://doi.org/10.2147/RRTD.S75809

Wilkinson, S.C., Maas, W.J.M., Nielsen, J.B., Greaves, L.C., van de Sandt, J.J.M., Williams, F.M., 2006. Interactions of skin thickness and physicochemical properties of test compounds in percutaneous penetration studies. Int. Arch. Occup. Environ. Health 79, 405–413. https://doi.org/10.1007/s00420-005-0056-5

Yu, Y.-Q., Yang, X., Wu, X.-F., Fan, Y.-B., 2021. Enhancing Permeation of Drug Molecules Across the Skin via Delivery in Nanocarriers: Novel Strategies for Effective Transdermal Applications. Front. Bioeng. Biotechnol. 9. https://doi.org/10.3389/fbioe.2021.646554

Yun, Y.E., Jung, Y.J., Choi, Y.J., Choi, J.S., Cho, Y.W., 2018. Artificial skin models for animal-free testing. J. Pharm. Investig. 48, 215–223.

https://doi.org/10.1007/s40005-018-0389-1

Zimoch, J., Zielinska, D., Michalak-Micka, K., Rütsche, D., Böni, R., Biedermann, T., Klar, A.S., 2021. Bio-engineering a prevascularized human tri-layered skin substitute containing a hypodermis. Acta Biomater. 134, 215–227. https://doi.org/10.1016/j.actbio.2021.07.033

6. DISCUSSÃO GERAL

Como já mencionado no Capítulo I, a TST é o hormônio responsável pelo desenvolvimento e manutenção das características masculinas. No decorrer da vida, e principalmente após os 40 anos de idade, a produção da TST começa a diminuir. Isso pode estar relacionado com uma produção inefetiva do hormônio pelos testículos ou também por uma deficiência na produção dos precursores hormonais. Essa condição é conhecida como hipogonadismo e está associada a diversos sintomas clínicos, sexuais e psicológicos. Para reduzir ou evitar efeitos indesejáveis da redução hormonal, diferentes formulações a base de TST têm sido desenvolvidas a fim de restaurar os seus níveis fisiológicos, incluindo diferentes rotas de administração (MILLS; MAGNUSSON; CROSS, 2006). Os sistemas transdérmicos têm sido priorizados devido a maior adesão do paciente ao tratamento como são não invasivos e indolores (BAERT et al., 2012).

Embora as formulações transdérmicas sejam mais convenientes para o paciente, a TST apresenta limitações biofarmacêuticas que desafiam o desenvolvimento de novos sistemas. A TST é um hormônio relativamente lipofílico, com uma massa molar e log P intermediário, o que contribui para uma alta afinidade pela pele. A sua afinidade ao tecido também ocorre devido à presença de receptores androgênicos no tecido cutâneo (CHEN; THIBOUTOT; ZOUBOULIS, 2002). Dentre as estratégias utilizadas para promover uma entrega de TST em concentrações adequadas, destaca-se o uso de promotores de absorção. Estas substâncias agem modificando a estrutura da pele, seja nos domínios lipídicos ou proteicos, reduzindo, assim, as propriedades de barreira do estrato córneo e facilitando a absorção.

Há um número apreciável de estudos de permeação cutânea com a TST na literatura. A maioria desses estudos selecionou a TST como fármaco modelo para investigar o perfil de permeação de moléculas com características lipofílicas. Nesse sentido, o capítulo II apresentou um artigo de revisão que trata da avaliação do comportamento da TST em ensaios de permeação que utilizaram células de difusão do tipo Franz diante de diferentes condições experimentais.

De forma resumida, a análise dos artigos concluiu que as diferentes etapas de um estudo de permeação com moléculas lipofílicas devem ser realizadas de forma criteriosa. Por exemplo, para o desenvolvimento de estudos de permeação com a TST, a

etapa prévia de avaliação da solubilidade garante que o fármaco esteja solúvel tanto nos meios receptor quanto no doador e não haja subestimação do coeficiente de permeação. Nesse contexto, a utilização de agentes solubilizantes ou cossolventes se torna necessário sempre que o fármaco apresentar uma baixa solubilidade em água. Nos estudos analisados, os cossolventes mais utilizados foram o etanol e o propilenoglicol, os quais também podem atuar como promotores de permeação. Este estudo de revisão também ressalta que deve ser estabelecida uma concentração máxima de solvente para não alterar a integridade do tecido e, desta forma, se observar um aumento de permeação inespecífico (dados de fluxo e coeficiente de permeação superestimados).

A seleção da fase doadora mostrou impactar significativamente no perfil de permeação, uma vez que se observou uma redução da taxa de permeação da TST com o aumento da sua solubilidade nesta fase. Dessa forma, os estudos que utilizaram o propilenoglicol (no qual a TST é menos solúvel) apresentaram uma maior taxa de absorção da TST comparado ao etanol. Além disso, a condição de supersaturação nem sempre vai ser favorável nos estudos de permeação e deve ser avaliada com cautela pelos pesquisadores. Em alguns casos pode ocorrer a precipitação do fármaco no meio supersaturado, sendo necessária a inclusão de agentes que inibem a precipitação. Outra importante análise dessa revisão traz que o uso da pele inteira pode subestimar a quantidade de fármaco efetivamente permeada. Os estudos em que se utilizou uma espessura de aproximadamente 400 μm resultou em maiores taxas de permeação comparativamente aos estudos com as peles inteiras. Isso sugere que os experimentos com fármacos lipofílicos devem ser realizadas preferencialmente com pele dermatomizada para evitar retenção na camada da derme.

Outro parâmetro analisado foi o modelo de membrana. A taxa de permeação variou entre os estudos que usaram diferentes tipos de pele animal, destacando uma grande diferença entre as peles de roedores e a humana para vários tipos de moléculas lipofílicas e hidrofílicas. Ainda, a baixa taxa de difusão da TST através de pele humana e suína levou os pesquisadores a estender o tempo experimental, sendo que a maior parte dos experimentos foi realizada em 24 horas ou em um intervalo ainda maior quando esses tecidos foram usados. Assim, um maior tempo experimental deve ser considerado quando do uso destes tecidos, porém, os pesquisadores devem estar atentos aos efeitos dos solventes e outras condições que afetam a integridade dos tecidos. Por fim, observou-se que a maior parte dos estudos utilizou a cromatografia líquida de alta

eficiência para a quantificação da TST em diferentes matrizes biológicas. Acetonitrila e o metanol foram os solventes mais empregados para romper a ligação do hormônio com as proteínas e precipitação delas. Poucos estudos analisados apresentam dados de retenção cutânea da TST. Ainda assim, foi possível identificar que a TST fica retida em maior concentração no estrato córneo e na epiderme (são mais lipofílicos) do que na derme, respeitando a natureza mais lipofílica da molécula. É importante mencionar que a presença de receptores androgênicos nas glândulas sebáceas, na região dos folículos e na papila dérmica dos queratinócitos pode favorecer a sua retenção, porém, há uma carência de estudos de avaliação da retenção cutânea da TST e seu consequente efeito nas camadas da pele.

Considerando esta lacuna, o capítulo III avaliou o perfil de permeação e retenção da TST nas camadas de pele humana e investigou o efeito da presença de diferentes terpenos (α-bisabolol, carvacrol e mentol) na promoção da permeação. Estes agentes atuam extraindo ou fluidizando os lipídeos, enfraquecendo a barreira do estrato córneo. Por terem origem natural, apresentam baixa toxicidade, além de possuírem atividades secundárias interessantes para tratamentos de ação local como ação anti-inflamatória e antimicrobiana.

A primeira etapa do estudo determinou o tempo necessário para realizar o experimento, visto que a TST possui baixa taxa de permeação. A TST foi detectada em nível quantificável apenas após 16 horas. Com relação à retenção cutânea, houve um aumento de 2 a 3 vezes na quantidade de hormônio nas camadas da pele quando os ensaios passaram de 24 para 36 horas. Nesse sentido, o tempo de 36 horas foi o mais adequado, pois permitiu a construção de um perfil de permeação e assim a determinação dos parâmetros de permeação, como o fluxo, coeficiente de permeação, tempo de latência, bem como a quantificação da TST nas camadas da pele. Na segunda etapa do estudo, os dados fornecidos sugeriram que os terpenos melhoraram a permeação da TST quando comparados ao controle. Dentre os terpenos testados, o carvacrol foi o que resultou em um maior aumento de fluxo e coeficiente de permeação. Embora os outros tratamentos também tenham sido efetivos, essa diferença não foi estatisticamente significante. O carvacrol também foi o único terpeno que reduziu o tempo de latência comparado ao controle, sugerindo um início mais rápido da atividade terapêutica na presença desse agente. Além disso, o carvacrol promoveu uma maior retenção da TST

na camada mais superficial da epiderme, corroborando com os resultados de maior permeação.

Na sequência, análises de FT-IR e DSC foram realizadas para identificar os principais mecanismos de promoção da permeação dos terpenos. Como a TST possui preferência pela rota intercelular de transporte devido a sua natureza lipofílica, promotores que alterem mais os domínios lipídicos são mais efetivos no aumento do fluxo de permeação. A análise no FT-IR identificou desordens em domínios lipídicos do estrato córneo. O carvacrol foi o terpeno que promoveu alterações mais significativas no estrato córneo (maior desordem nas cadeias lipídicas), o que está de acordo com a maior permeação e retenção da TST na presença deste agente. Nos ensaios de DSC, as mudanças térmicas mais importantes também apontaram para uma maior extração de lipídios do estrato córneo pelo carvacrol, especialmente próximo a 65°C. O mentol e carvacrol apresentaram pequenas reduções em temperatura próxima a 80°C, sugerindo formação de complexos entre proteínas e lipídeos e, com isso, maior rompimento da bicamada lipídica. As amostras com carvacrol e o α -bisabolol mostraram maior redução endotérmica que o mentol nessa região, indicando maior fluidização. Ainda, cabe ressaltar que acima de 90°C inicia-se um processo de desnaturação das proteínas. Este evento pode ser detectado em amostras de tecido onde houve extração de lipídios e foi observado com maior intensidade para o α-bisabolol e o carvacrol, o que pode estar relacionado com a extração lipídica.

Por fim, o conjunto dos dados do estudo de permeação e da avaliação mecanística sugere que, embora o carvacrol e o mentol apresentem estruturas químicas e propriedades físico-químicas semelhantes, há diferenças importantes no tipo e intensidade de interações entre estes compostos e as estruturas lipídicas e proteicas da pele. De fato, isso é observado nos diferentes perfis obtidos na permeação da TST. O carvacrol se mostrou mais efetivo no aumento de ambas permeação e retenção da TST por apresentar maior afinidade por domínios lipídicos. Ainda, o carvacrol e o α-bisabolol mostraram semelhanças no infravermelho, porém, o carvacrol apresentou maior fluxo de permeação. Isso pode ser explicado pelas características físico-químicas desses compostos já que o α-bisabolol apresenta maior log P, o que resulta em maior interação com as camadas superficiais da pele. Além disso, o α-bisabolol possui maior massa molar, o que pode reduzir a taxa de difusão do composto através da pele.

Os resultados do capítulo III apontam o carvacrol como o terpeno que gera efeitos mais expressivos na estrutura da pele e, por isso tende a promover maior permeação de compostos lipofílicos. Assim, a incorporação do carvacrol como agente promotor em formulações de uso tópico contendo TST pode ser uma estratégia para aumentar os níveis de TST na circulação sanguínea. Ainda, o carvacrol apresenta atividade antioxidante, pode melhorar a qualidade dos espermatozoides (fator relevante para os pacientes como hipogonadismo), além de suas funções bactericida e antifúngica que podem auxiliar na conservação das formulações. Outro ponto interessante é que esse terpeno não interage com a CYP3A4, não atrapalhando assim a metabolização de xenobióticos e, consequentemente, não ocasionando interações medicamentosas.

O capítulo II apresentou uma análise detalhada dos principais modelos de membranas usadas nos estudos de permeação cutânea da TST, sendo que os modelos animais foram os mais empregados. Dentro desse contexto, o capítulo III apresentou um estudo de permeação com a TST usando pele humana, a qual tem vantagens inerentes a sua constituição. No entanto, questões éticas e muitas vezes a difícil aquisição da pele humana podem inviabilizar a realização dos experimentos. As membranas sintéticas são uma alternativa ao uso de pele. Além disso, há um esforço contínuo das agências regulatórias para que as pesquisas evitem a utilização de animais. Contudo, embora a utilização das membranas sintéticas e de cultura celular seja reconhecida pela OECD para estudos de toxicologia, ainda continua sendo um desafio a utilização dessas membranas nos ensaios de permeação. Nesse cenário, o capítulo IV apresentou uma revisão atualizada sobre a aplicação de modelos de membranas em estudos de permeação cutânea para moléculas com diferentes propriedades físico-químicas.

O capítulo IV foi baseado na comparação de dados de estudos de permeação usando membranas biológicas, sintéticas ou cultura de células a partir de dois fármacos modelos: a cafeína (CF), um composto hidrofílico e a TST, um composto lipofílico. A CF e a TST são as moléculas mais usadas em estudos de permeação cutânea com novos modelos de membrana a fim de validá-los. A análise desses estudos mostrou que a espessura das membranas biológicas (pele animal ou humana) afetou significativamente a absorção da TST, sendo dez vezes maior nos estudos que utilizaram espessuras menores de pele. No entanto, esse parâmetro não afetou a permeação da CF. Isso confirma a necessidade de um maior rigor com relação à espessura de membranas

biológicas para se alcançar resultados mais similares a situação fisiológica, priorizando-se tecidos dermatomizados em ensaios com moléculas lipofílicas.

As membranas sintéticas geram resultados mais reprodutíveis que as membranas biológicas, além de permitirem uma maior compreensão da organização do estrato córneo, bem como do impacto de cada componente nas propriedades de barreira. Estas membranas podem ser utilizadas alocando-as em um dispositivo de placas de 96 cavidades chamado PAMPA ou na interface dos compartimentos que compõe as câmaras de difusão. Os estudos apontaram maior correlação entre PAMPA e a pele humana para a TST que CF. A utilização de membranas sintéticas no aparato PAMPA parece ser mais interessante durante a triagem de novos compostos. De modo geral, estudos mostram que as membranas sintéticas podem ser consideradas como modelos de pele preferencialmente nas seguintes situações: quando o estrato córneo é a principal barreira; o composto não sofre metabolização enzimática nem apresenta receptores na pele; quando não há a necessidade de utilização de promotores de permeação que podem interagir com a membrana e danificá-la e quando os ensaios *in vivo* podem ser facilmente executados para validação dos dados.

Com relação às membranas de cultura celular, diferentes tipos de células humanas tem sido cultivadas em camadas sobre uma matriz polimérica. Essas podem ser desenvolvidas visando representar somente a epiderme ou a pele completa. Uma vantagem do uso de células é que os modelos podem expressar enzimas metabólicas e outras estruturas importantes, o que torna o experimento mais próximo a situação fisiológica. No entanto, os estudos mostram que esse modelo é mais permeável que as peles de humano e suína ao apresentarem maior K_p e menor *lag time*. Essa diferença pode ser decorrente da diferente composição lipídica na membrana de cultura de células em comparação com a membrana biológica em proporção. Outra possível explicação é com relação às diferenças obtidas no empacotamento e organização lipídica do estrato córneo das membranas cultivadas em cultura. Além disso, esse tipo de modelo não é adequado para a avaliação de transporte pelos apêndices, principalmente para moléculas hidrofílicas como a CF e os esteroides como a TST.

O desenvolvimento de estudos de permeação para avaliação do desempenho de moléculas através da pele exige avaliação criteriosa dos diferentes parâmetros envolvidos. De modo geral, cada etapa deve ser analisada com relação ao tipo de

fármaco, membrana usada e seleção de fluidos. O capítulo II desmembrou cada etapa e apresentou a importância da seleção adequada desses parâmetros para moléculas com características lipofílicas. Já o capítulo III apresentou um estudo da avaliação de terpenos, que são promotores que impactam preferencialmente em domínios lipídicos da pele humana, e os consequentes efeitos na permeação da TST. A análise mecanística gerou dados que apoiam o carvacrol como um promotor promissor para uso em produtos tópicos que necessitam aumentar a taxa de permeação de fármacos para obtenção de níveis terapêuticos, como o caso da TST. Por fim, a revisão apresentada no capítulo IV analisou as vantagens e limitações de cada modelo de membrana (biológica e não biológica) na permeação de compostos, uma vez que é crescente o incentivo ao não uso de animais. Assim, modelos sintéticos e de cultura celular vem sendo aprimorados na busca de resultados cada vez mais próximos aos estudos *in vivo*. De modo geral, a seleção da membrana deve considerar as características da molécula do fármaco em análise, além de manter um rigoroso controle de qualidade e especificação de procedimentos para garantir a reprodutibilidade, já que esses ensaios são fundamentais durante o desenvolvimento de novas formulações transdérmicas.

7. CONSIDERAÇÕES FINAIS

– Deve-se alcançar um equilíbrio entre solubilidade e permeação para maximizar a taxa de transporte de fármacos pouco solúveis como a TST através da pele;

– Uso de etanol aumenta a solubilidade, porém, reduz a taxa de permeação da TST;

– Um grau de supersaturação ideal deve ser encontrado para maximizar a permeação de fármacos e evitar fenômenos de precipitação dos mesmos;

– A derme atua como uma barreira reduzindo a partição da TST para o meio receptor;

– Priorizar tecidos dermatomizados em ensaios com fármacos lipofílicos para reduzir a retenção dos mesmos;

– Diferenças substanciais no transporte do hormônio foram encontradas entre as espécies animais analisadas e membranas artificiais;

– Os terpenos se mostraram efetivos no aumento da permeação da TST pela pele;

– Dentre os terpenos analisados, o carvacrol e o mentol foram o mais e menor efetivo como promotores de absorção, respectivamente;

– O carvacrol e o α-bisabolol interagiram mais com os domínios lipídicos do que o mentol;

– Como a TST prefere rotas de transporte passivo, uma maior desordem dos domínios lipídicos tem impacto direto na permeação;

– Modelos de pele reconstruída (aqueles que envolvem cultivo celular) são mais permeáveis que pele *in natura*;

– As membranas sintéticas e de cultura celular não preveem o transporte de ativos pelos apêndices;

– Modelos de pele reconstruída apresentam a vantagem de possibilitar a avaliação metabólica nos ensaios de permeação diferente das membranas sintéticas;

– Embora haja esforços para se obter a mesma composição lipídica entre os modelos de pele reconstruída e as biológicas, ainda há grandes diferenças na organização lipídica dos tecidos;

– Deve-se ter cautela com a utilização de solventes para se alcançar as condições "sink" durante a permeação de moléculas lipofílicas devido à possibilidade de solubilização dos lipídios, afetando as propriedades de barreira das membranas artificiais ou em modelos de pele reconstruída;

– As peles humanas e suínas ainda são os modelos mais representativos para os estudos de permeação.

REFERÊNCIAS

BAERT, Bram et al. Increase of the transdermal penetration of testosterone by miconazole nitrate. **Archives of Pharmacal Research**, [S. l.], v. 35, n. 12, p. 2163–2170, 2012. Disponível em: https://doi.org/10.1007/s12272-012-1214-4

CHEN, WenChieh; THIBOUTOT, Diane; ZOUBOULIS, Christos C. Cutaneous Androgen Metabolism: Basic Research and Clinical Perspectives**. Journal of Investigative Dermatology**, [S. l.], v. 119, n. 5, p. 992–1007, 2002. Disponível em: https://doi.org/10.1046/j.1523-1747.2002.00613.x

MILLS, P. C.; MAGNUSSON, B. M.; CROSS, S. E. The effects of vehicle and region of application on in vitro penetration of testosterone through canine skin. **Veterinary Journal**, [S. l.], v. 171, n. 2, p. 276–280, 2006. Disponível em: https://doi.org/10.1016/j.tvjl.2004.11.013