A stepwise protocol for drug permeation assessment that combines heat-separated porcine ear epidermis and vertical diffusion cells

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Abstract

After decades long absence of an official consensus on the most appropriate evaluation method for *in vitro* skin performance of topical semisolid drugs, United States Pharmacopoeia (USP 39) finally suggested three types of testing equipment; however, all these provide data on drug release using inert synthetic membranes. Considering the need for a readily available membrane that would be more structurally similar to human skin, this paper provides a detailed protocol of a method for drug permeation assessment that uses heat-separated porcine ear epidermis and modified Franz diffusion cells. Phases that were shown to be critical for variability of the results are identified (*e.g.*, membrane preparation), and process parameters optimized. Applicability of the method was tested on four cream samples loaded with aceclofenac as a model drug. Sample compositions were designed in such a way to provide „large" variations (variation of the main stabilizer: natural-origin *versus* synthetic emulsifier) and relatively „minor" variations (co-solvent variation: none/isopropanol/glycerol). The developed protocol is a straightforward and reliable *in vitro* test for the evaluation of rate and extent of drug delivery into/through the skin. Moreover, this protocol may be routinely applied even in averagely equipped laboratories during formulation development or preliminary bioequivalence assessment of generic topical semisolids.

Keywords: dermal drug delivery, rate and extent of drug permeation, hydrophilic creams, aceclofenac.

Available online at the Journal website: http://www.ache.org.rs/HI/

TECHNICAL PAPER

UDC 615.45:615.03:54

Hem. Ind. **72** (1) 47–53 (2018)

The number of generic topical semisolid drugs on the world market is somewhat limited, mainly due to problems related to establishing bioequivalence with a suitable reference [1]. Since the target site of action for the majority of topical drugs is the skin itself or subcutaneous tissue, establishing bioequivalence usually assumes the need for clinical trials, which are associated with significant result variability and poor sensitivity in discerning potential differences among similar formulations [1–4]. Therefore, in order to facilitate development of high-quality generic topical semisolid drugs, regulatory agencies, specifically FDA (Food and Drug Administration) and EMA (European Medicines Agency), show interest in development of alternative approaches [5,6]. Namely, after comparative analysis of physical, chemical and microstructure-related critical quality attributes of the test and reference preparations, evaluation of bioequivalence may be performed using a combination of different *in vitro* and *in vivo* pharmacokinetics-based methods.

The onset, intensity and duration of a therapeutic response after dermal application of semisolid dosage forms depend on the relative efficacy of several successive processes: drug release from the carrier/base, drug penetration into and diffusion across the *stratum corneum* as the rate-limiting skin layer, and subsequent drug permeation through viable epidermis and/or dermis to the drug-specific receptor [2]. Therefore, in order to rationalize both time and funds available for formulation development of topical drugs, prior to embarking upon time-consuming and expensive *in vivo* studies, *in vitro* tests are usually performed [7]. While the European Pharmacopoeia (Ph. Eur. 9.0) does not elaborate on *in vitro* methods for drug release testing from semisolid dosage forms [8], US Pharmacopoeia (USP 39) provides a detailed description of three applicable methods (vertical diffusion, immersion and flow-through cells) coupled with inert, porous, synthetic membranes [9]. Although we agree that synthetic membranes may be reliable enough to provide time-dependent drug release/liberation profiles, the conclusions drawn from such data often cannot relate to the actual *in vivo* performance. This is usually due to the inability of synthetic membranes to simulate a

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Paper received: 26 July 2017

Paper accepted: 03 November 2017

https://doi.org/10.2298/HEMIND170726019P

complex interplay that occurs between the skin and the formulation excipients upon application [10]. Therefore, we strived to define a detailed protocol of an *in vitro* evaluation approach for drug permeation assessment from topical semisolid preparations involving vertical (Franz) diffusion cells, and a membrane that would be more structurally similar to human skin.

Although isolated human skin is considered a „gold standard", sufficient quantities are generally hard to obtain for both ethical and financial reasons (*e.g.*, after aesthetic surgery) [1,11,12]. Survey of the available literature revealed that similar methods were already used for testing (trans)dermal drug delivery of different model drugs, and utilize membranes of various origin and complexity (skin isolated from different animal species, reconstructed human skin equivalents, *etc*.) [11,13–15]. For that reason, Organisation for Economic Co-operation and Development (OECD) published a guideline stating the suitability of porcine ear skin for percutaneous absorption studies, due to the demonstrated structural, morphological and permeability similarities to the human skin [16–18]. Apart from that, recent findings even confirmed certain immunological similarities of porcine and human skin [19].

However, in spite of the numerous efforts in defining guidelines for *in vitro* percutaneous absorption testing, studies conducted so far imply that the obtained results may be influenced by a number of factors [16,20,21]. Therefore, each and every laboratory was left to define more precise experimental conditions for performing such studies. Additionally, papers in the field failed to disclose a detailed methodology or, in our opinion, misinterpreted some critical protocol parameters, leading to results that could not be easily reproduced.

For that reason, the aim of our work was to define a detailed protocol for an *in vitro* drug permeation study through heat-separated porcine ear skin epidermis using Franz diffusion cells that could be routinely used in laboratory settings. Implementation of such a protocol should enable direct comparison of both rate and extent of drug delivery into/through the skin from the investigated formulations. Special emphasis was given to the optimization of the membrane preparation protocol, since pig ears are relatively available biological materials that could be modelled into an alternative membrane for this type of studies.

EXPERIMENTAL

Existing publications on *in vitro* drug permeation studies that rely on alternative membranes, suggest rather complicated and time-consuming protocols that are often lacking data relating to optimal conditions for preparation of such membranes [4,22–25]. We opted for heat-separated porcine ear epidermis as a suitable membrane for this type of experiments, considering that drugs enter systemic circulation in superficial dermal layers. Consequently, the presence of the entire hydrophilic dermis in *in vitro* settings would serve as an artificial barrier for poorly soluble drugs. Therefore, isolated epidermis obtained by a suitable method should be a promising model for *in vitro* drug permeation testing [4,20].

Our study was divided into three main phases: *i*) optimization of the protocol for heat-separated porcine ear epidermis preparation, *ii*) assessing compatibility of the prepared membrane with the modified Franz diffusion cells system, and *iii*) quantification and calculation of thus obtained permeation data. The protocol was tested for hydrophilic creams as the most frequently used topical semisolid dosage forms.

Sample design with the purpose of protocol applicability assessment

Tested creams were either stabilized with a sugar (*i.e.*, natural-origin) emulsifier of the alkyl polyglucoside type (cetearyl glucoside and cetearyl alcohol, Sepineo^{TM} SE 68, Seppic, France; samples labelled APG), or pertained synthetic non-ionic emulsifiers (polysorbate 60 and cetearyl alcohol, Sigma Aldrich). Such a variation of the main emulsion system stabilizer significantly affects the cream microstructure, and was regarded as a „large" variation during the method optimization. It should be noted that the formulation stabilized with synthetic emulsifiers was in fact a pharmacopoeial quality non-ionic hydrophilic cream [26], that is often used as a reference sample (thus labelled R) [27–30]. Additionally, protocol sensitivity to discern „minor" differences in the investigated formulations composition was tested with samples based on the aforementioned natural-origin emulsifier that were varied in presence or absence of certain co-solvents and potential penetration enhancers. This variation was designed at three levels (*i.e.*, no co-solvent added (sample APG), addition of isopropanol (sample APG+Ipa) or glycerol (sample APG+Gly)). All the samples were loaded with 1 mass% of aceclofenac as a model drug.

Membrane (heat-separated porcine ear epidermis) preparation

This experimental phase involved the following equipment: ears of domestic white pigs approximately 4 months old, 0.9 mass% sodium chloride solution, scalpel, electrical trimmer HC 5410/15 (Philips, Netherlands), tweezers, an in-house made spherical cutter (alternatively surgical scissors), phosphate buffer pH 7.4 (2.38 g $Na_2HPO_4 \cdot 12H_2O$, 0.19 g KH_2PO_4, 8.0 g NaCl in 1 L of highly-purified water), magnetic stirrer with a heating function (IKA RH basic 2, IKA, Germany), cotton pads and disposable latex gloves.

In vitro permeation study using Franz diffusion cells and heat-separated porcine ear epidermis

The second experimental stage required the following equipment: modified Franz diffusion cells with a surface of 2.01 cm^2 available for diffusion and 12 mL volume of the receptor compartment (Gauer Glas, Germany), metal clamps N°28 INOX 18/8 that pinch flat joints of donor and receptor compartments, Glisseal® HV (Borer Chemie, Switzerland) for sealing the joints of two cell compartments, ultrasonic bath (Sonorex RK102H, Bandelin, Germany), phosphate buffer pH 7.4 (composition given in section 2.2.), Parafilm® "M" (Bemis, USA), balance (Sartorius, Germany), tweezers with flattened shovels, 1 mL syringe with 21Gx43/4" (0.8 mm×120 mm) needle, investigated samples (aceclofenac 1 mass% loaded hydrophilic creams differing in base composition).

Quantification and calculation of drug permeation profiles

Precise quantification of aceclofenac in the sampled aliquots is usually conducted using a suitable HPLC method that may be coupled with different detectors. In our case, an UHPLC–MS/MS method was used as described in elsewhere [30]. Since the protocol includes a biological material (the membrane), the method validation was carried out according to FDA's guideline for bioanalytical methods [31].

RESULTS AND DISCUSSION

After varying multiple parameters such as porcine skin cleaning, storage and defrosting conditions (temperature, duration), the following protocol for porcine ear-based membrane preparation was defined.

In order to preserve the skin barrier integrity, ears should be taken immediately after slaughtering and washed carefully under cold tap water. The skin of the outer side of porcine auricle is then carefully separated from cartilage by a scalpel. Thus obtained full thickness skin is cleaned by cotton pads impregnated with isotonic sodium chloride solution, dried using a cotton cloth, protected with an aluminium foil and stored at –20 °C, but not longer than a month. During the development of the protocol, transepidermal water loss (TEWL) of porcine ear skin was frequently monitored by a Tewameter® TM210 (Courage+Khazaka, Germany). The obtained values of this biophysical parameter showed that the aforementioned temperature and duration of storage do not affect the skin barrier integrity. On the day of the experiment, after defrosting at room temperature (22±2 °C, TEWL values are in the range of 10–20 g m^{-2} h^{-1}), the hair is carefully cut to 0.5 mm length by using an electrical trimmer, and the skin is cut in round pieces of 25 mm diameter. Skin cutting may be performed by common surgical scissors; alternatively, a custom-made cutter with a spherical blade may be used (Figure 1c), since it facilitates this stage and provides uniform size. The skin is then immersed in purified water heated to 60 °C for 90 s after which the epidermis is carefully removed from the dermis by tweezers. Isolated epidermis is inspected for physical damage with a magnifying glass, transferred to a Petri dish with phosphate buffer pH 7.4 and left to hydrate for 1 h.

After successful membrane preparation, its suitability for application in modified Franz diffusion cell setup was assessed. Firstly, the joints of donor and receptor compartments of diffusion cells are sealed using a small amount of Glisseal® HV in order to prevent water entry into the cell during the experiment. A stirring magnet is placed in each receptor compartment that is subsequently completely filled with phosphate buffer pH 7.4 that was previously submitted to sonication for 30 min (for the sake of air bubbles removal) and heated to 32 °C (average skin surface temperature). Porcine ear epidermis is then placed with utmost care between the donor and receptor compartments (*stratum corneum* facing the donor part) using a flattened shovels tweezer, and the compartments are fixed by a clamp. Receptor parts of the cells are immersed into a water bath at 32 °C in order to equilibrate the

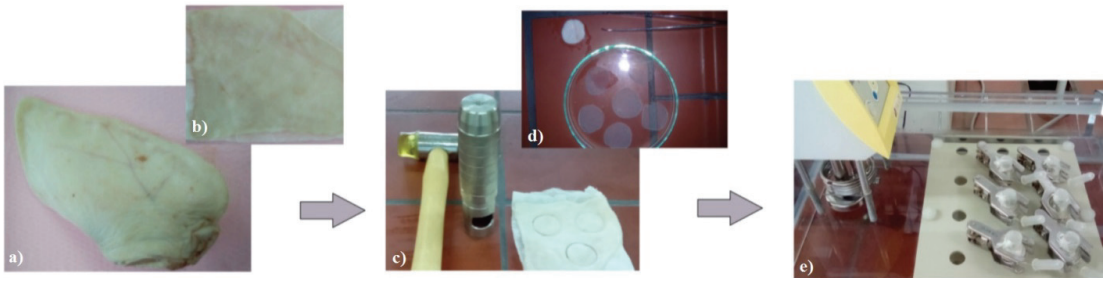

Figure 1. Schematic representation of the protocol for the preparation of heat-separated porcine ear epidermis that precedes the actual drug permeation assessment: a) the photograph of a porcine ear, as received after slaughtering, b) the photograph of the outer side of porcine auricle skin when separated from cartilage, c) the use of the spherical cutter ensures uniform membrane dimensions, d) the photograph of the isolated epidermis placed in a Petri dish versus a full thickness skin membrane, and e) the final setup of the modified Franz diffusion cells.

membranes under constant magnet stirring at 500 rpm. After 30 min, 1 g of each sample is weighted and uniformly applied to the membrane surface. The donor compartment is then occluded using Parafilm®. Drug permeation through the heat-separated porcine ear epidermis is investigated over the next 30 h to achieve the steady-state as well as to avoid inevitable degradation of the skin barrier. The temperature and stirring speed are kept constant. At previously defined time points (in our case: 2, 4, 6, 8, 20, 22, 24, 26, 28 and 30 h), 600 µL aliquots are sampled by a needle and syringe with special care to prevent air bubbles formation. After each sampling, the system is refilled with an equal volume of heated, degassed phosphate buffer, in order to maintain sink conditions. Aceclofenac concentration in sampled aliquots is then determined by an appropriate UHPLC–MS/MS method.

Output of such analytical methods is usually presented as drug concentration (*e.g.*, µg of aceclofenac per mL) and need to be further corrected for the sake of proper results discussion. The stepwise calculation leading to the actual drug permeation profile is disclosed henceforth.

The obtained drug concentrations are first corrected to the buffer volume in receptor compartments (12 mL) and aliquot volume sampled in predefined time points (600 µL), and then divided with the surface available for diffusion (2.01 cm^2), in order to finally be expressed as cumulative amounts of drug permeated per surface unit Q (µg/cm^2):

$$Q_1 = \frac{C_1 V_c}{P} \quad (1)$$

$$Q_2 = \frac{(C_2 V_c + C_1 V_u)}{P} \quad (2)$$

$$Q_3 = \frac{(C_3 V_c + C_1 V_u + C_2 V_u)}{P} \quad (3)$$

where Q_1, Q_2, Q_3, *etc.*, represent aceclofenac amount that permeated per membrane (heat-separated epidermis) surface unit at times t_1, t_2, t_3, *etc.*; C_1, C_2, C_3, *etc.* – drug concentrations (µg of aceclofenac per mL) in aliquots sampled at appropriate time points (t_1, t_2, t_3, *etc.*), determined *via* UHPLC–MS/MS method; V_C – volume of the diffusion cell receptor compartment; V_U – volume of the phosphate buffer sampled at times t_1, t_2, t_3, *etc.*; and P – surface of the diffusion cell available for drug diffusion.

For better clarity, the obtained results may be further graphically presented as time (t_i) dependent plots of the cumulative drug amount (Q_i) that permeates through the surface unit of heat-separated porcine ear epidermis (Figure 2). The obtained permeation profiles enable direct comparison of drug (aceclofenac) delivery efficacy into/through the skin from various formulations.

In order to fully characterize drug permeation process through heat-separated porcine ear epidermis from the selected formulations, besides permeation profiles, values of the following parameters are also calculated: total amount of drug permeated through the membrane at the end of the experiment (Q_{30h}), steady-state flux (J_{ss}) and permeability coefficient (K_p).

Steady-state flux (J_{ss}) of the drug through the skin is determined from the linear portion of the slope of the cumulative amount of the drug that permeates through the membrane surface unit *versus* time plot, for each investigated formulation and each used diffusion cell. Linear portion of the curve is obtained using linear regression analysis of a sufficient number of measuring points in the steady-state phase. After this, permeability coefficient (K_p) is calculated as follows [12]:

$$K_p = \frac{J_{ss}}{C_0} \quad (4)$$

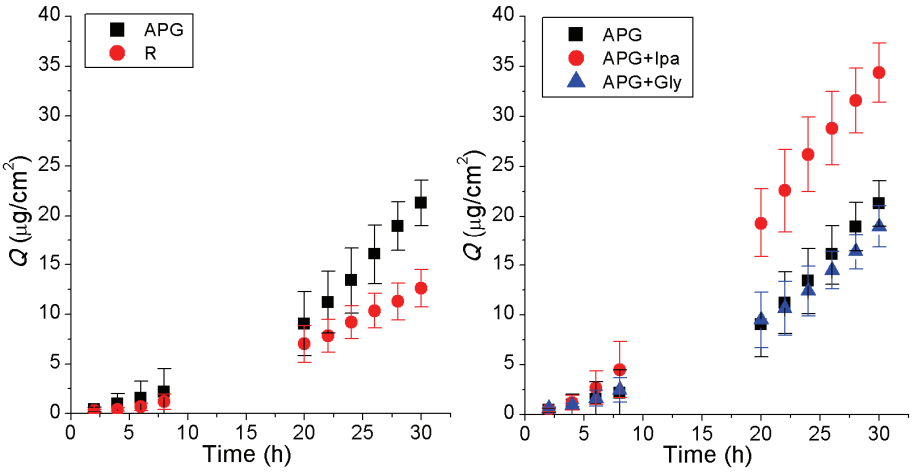

Figure 2. Comparative representation of aceclofenac permeation profiles from the investigated samples: a) comparison of samples with „large" variations in delivery system microstructure and b) comparison of samples with „minor" variations in formulation composition (mean values ± standard deviations, n = 6).

where J_{ss} is the steady-state flux of the drug through the skin (μg/(cm^2 h)), and C_o initial drug (aceclofenac) concentration in the tested formulation (μg/mg).

Aceclofenac permeation profiles shown in Figure 2, as well as the values of steady-state flux, permeation coefficient and the total amount of aceclofenac that permeated through the skin at the end of the experiment imply that the presented protocol is successful in discerning differences in aceclofenac availability from the investigated hydrophilic creams. As expected, the protocol was able to point towards the differences in aceclofenac permeation parameters from APG *vs.* R samples. In fact, the difference in the two samples' microstructure has resulted in a nearly two-fold higher J_{ss}, Q_{30h} and K_p values ($p < 0.05$) in favour of the aceclofenac sample stabilized with the natural-origin emulsifier (calculated values for sample APG were 1.22± ±0.19 μg/(cm^2 h), 21.04±2.04 μg/cm^2 and 0.12±0.02 mg/(cm^2 h), respectively; while the corresponding values for sample R were 0.57±0.09 μg/(cm^2 h), 12.62±1.88 μg/cm^2 and 0.06±0.01 mg/(cm^2 h)). Admittedly, such substantial formulation variations (*i.e.*, „large" variations) could also be revealed via *in vitro* release tests equipped with synthetic membranes, such as polycarbonate ones our group has used in reference [30]. Naturally, the differences of the aforementioned permeation parameters among the samples that endured smaller composition variations (*i.e.*, co-solvent addition) were not as prominent but were still notable (J_{ss} was found to be 1.6-fold; Q_{30h} 1.8-fold and K_p 1.5--fold higher for the sample APG+Ipa as compared to the sample APG+Gly). In fact, the sample APG+Ipa provided a significant increase in aceclofenac permeation rate, Q_{30h} and permeation coefficient (1.50±0.08 μg/ /(cm^2 h), 34.36±2.97 μg/cm^2 and 0.15±0.01 mg/(cm^2 h), respectively), all $p < 0.05$ when compared to other three tested samples. Contrary to these findings, when synthetic membranes are used instead of heat-separated porcine ear epidermis, the impact of isopropyl alcohol as a permeation enhancer with multifaceted mechanism of skin interactions [32], could not be detected. In fact, aceclofenac release testing through polycarbonate membranes favoured the basic APG sample, and not APG+Ipa sample (release rate and extent attributed to the sample APG were significantly higher when compared to all other tested samples; $p < 0.05$) [30]. This is another obvious contribution of bio-derived membranes application in elucidation of slight differences in the assessed topical semisolids.

The obtained results suggest that the defined protocol is able to detect the influence of relatively small variations in formulation of topical semisolids on dermal availability of the incorporated drug. Namely, although the composition of the tested alkyl polyglucoside-based creams differ only in the addition of a co--solvent (basic sample without a co-solvent (APG), sample with isopropanol (APG+Ipa) and sample with glycerol (APG+Gly)), clear differences in the recorded rate and extent of aceclofenac permeation could be discerned when heat-separated porcine ear epidermis was used as a membrane. Additionally, the results obtained by using this method are satisfactorily correlated to *in vivo* data obtained by a tape stripping method on human volunteers (R^2 = 0.9802; data not shown), which asserts the suitability of the method in detection of potential differences among formulations of the same dosage form [30]. Therefore, the data obtained by the presented *in vitro* method could also contribute to rationalization of consecutive assessment of product bioequivalence.

CONCLUSIONS

A protocol envisioned for *in vitro* efficacy assessment of topical semisolid drugs is presented in detail. The added value of such a permeation study lies in the application of a membrane (heat-separated porcine ear epidermis) that is relatively easily obtained and structurally similar to the human skin. Applicability of the protocol was shown for: *i*) aceclofenac permeation study from hydrophilic creams of relatively simple composition stabilized with a natural-origin alkyl polyglucoside emulsifier *vs.* a reference pharmacopoeial cream stabilized with a synthetic non-ionic emulsifier; *ii*) direct comparison of rate and extent of aceclofenac permeation from the alkyl polyglucoside-based samples varying in addition of co-solvents/potential penetration enhancers (isopropanol, glycerol).

The presented protocol does not require expensive state-of-the-art equipment but rather relies on tools that may be found in every research laboratory focused on topical preparations. It is our opinion that researchers could benefit from such a protocol especially during formulation development, when selection should be made among a large number of formulation-candidates. This assumes that various formulations of the same dosage form (*e.g.*, creams, ointments or gels) could be submitted to a comparative assessment in order to reveal the most promising formulation regarding the expected relative bioavailability of the incurporated drug. Such a method could even serve for preliminary assessment of bioequivalence with generic topical semisolid drugs of the same dosage form.

Acknowledgements

The authors were supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia through the project TR34031.

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IZVOD

PROTOKOL ISPITIVANJA PERMEACIJE LEKOVITIH SUPSTANCI KROZ TOPLOTOM IZOLOVANI EPIDERMIS UHA SVINJE PRIMENOM VERTIKALNIH DIFUZIONIH ĆELIJA

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Nakon dugogodišnjeg nepostojanja zvaničnog stava o preporučenim metodama *in vitro* ispitivanja lekova polučvrste konzistencije za primenu na koži, Američka farmakopeja (USP 39) navodi vertikalnu difuzionu, imerzionu i protočnu ćeliju, kao tri vrste uređaja za ispitivanje. Iako sve navedene metode pružaju uvid u brzinu oslobađanja lekovite supstance iz ispitivanog preparata, one podrazumevaju upotrebu inertnih sintetskih membrana koje u dovoljnoj meri ne simuliraju kompleksnu strukturu kože i interakciju sa primenjenim preparatom. Imajući u vidu potrebu za lako dostupnom membranom koja bi po svojim karakteristikama bila sličnija humanoj koži, ovaj rad predlaže detaljan protokol metode za procenu permeacije lekova primenom epidermisa uha svinje izolovanog toplotom i modifikovane Francove difuzione ćelije. U radu su prepoznate kritične faze navedene metode (npr. priprema membrane), čijom se optimizacijom procesnih parametara smanjuje varijabilnost dobijenih rezultata. Osetljivost metode ispitana je na četiri jednostavna preparata tipa hidrofilnog krema sa aceklofenakom kao model lekovitom supstancom. Sastav preparata je koncipiran tako da obezbedi „velike“ razlike (npr. variranje osnovnog stabilizatora: emulgator prirodnog *versus* sintetskog porekla), kao i „male“ razlike u mikrostrukturi (npr. variranje korastvarača: bez korastvarača/izopropanol/glicerol), a sa ciljem njihove detekcije primenom razvijenog protokola. Razvijen protokol predstavlja jednostavnu i pouzdanu *in vitro* metodu za procenu brzine i obima isporuke lekovite supstance u/kroz kožu iz polučvrstih preparata. Ova metoda se može primeniti prilikom poređenja različitih formulacija u fazama razvoja novog leka ili preliminarne procene biološke ekvivalentosti generičkih preparata, čak i u laboratorijama prosečne opremljenosti.

Ključne reči: Dermalna isporuka lekovite supstance • Brzina i stepen permeacije leka • Hidrofilni kremovi • Aceklofenak