Research Article

DETERMINATION OF PERMEATION PATHWAY OF GENTAMICIN INTO PIG'S EAR SKIN

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ABSTRACT

Extensive research has been conducted in the recent years with a focus on drug administration via the skin for both topical and systemic drug delivery. Understanding the drug permeation through the skin is crucial for the development of an optimal product. In this study, the permeation of gentamicin through the pig's ear was evaluated. Both plugged and non-plugged skin was used. In non-plugged skin, the hair follicle orifices were open and a significant amount of gentamicin was detected. However, plugged skin, in which hair follicle orifices were artificially blocked, gentamicin can only penetrate to a less extent through interfollicular epidermis and possible through sweat glands. The study was performed using a Franz-type diffusion cell for 24 hours. The samples were withdrawn for each time interval and were analyzed by UV spectrophotometer. Cumulative amount of permeated gentamicin was compared using the drug concentration. The difference in the percentage of drug permeated through plugged and non-plugged was 44.8 %. Based on the obtained results, it can be concluded that the follicular route is an important route for the drug delivery through the skin.

Keywords: Gentamicin, Follicular route, Acne vulgaris, Pig's ear skin and Cosmetics.

INTRODUCTION

Skin is the outermost and largest organ of the body contributes 10 % of body weight and 1.7 m² of surface area. The most important function of the skin is it acts as a protective barrier of the body against external environment such as ultraviolet (UV) radiation, chemicals, allergens and microorganisms. The skin serves as an ideal administration sites for both local and systemic therapeutic compounds, though it provides effective barrier to penetration by external compounds¹. *In-vivo* study, the skin is able to regenerate continuously and thus is metabolically active². Skin made up of three structural layers known as, the epidermis (outer surface layer), dermis and subcutaneous tissues (deepest layer). Epidermis comprises of two different layers of epithelium, which are the viable epidermis and the stratum corneum. The viable epidermis serves as a hydrophilic layer with composition of 70 % water. On the other hands, the stratum corneum composed of only 13 % water serves as a hydrophobic layer. Hydrophilic compound is not able to penetrate easily across the hydrophobic stratum corneum. Hydrophobic compound can penetrate the stratum corneum but cannot enter the next layer of hydrophilic viable epidermis layer³. Dermis gives a mechanical strength to the skin as it composed of collagen fibrils embedded in mucopolysaccharide gel. In addition, dermis contains few embedded structures including blood and lymphatic vessels, hair follicles, sebaceous glands and sweat glands².

Figure 1: Structure of Skin

A topically therapeutic agent is able to penetrate through the skin via the stratum corneum⁴ and skin appendages including hair follicle⁵. The stratum corneum route can be divided into transcellular and intercellular routes. The transcellular route gives a direct penetration of the drug to cross through the stratum corneum. However, this route gives a significant resistance for drug permeation as the compound has to pass through both lipophilic and hydrophilic layer. Intercellular route is a more common pathway for drug entrance in which the compound moving between the corneocytes⁶. Recently, follicular penetration route has shown to be one of the efficient pathways for topically applied compounds⁷.

Figure 2: Penetration Pathways through the Skin

Pig skin has been a well-known experimental animal for many years. It serves as a good representative for human model skin due to similarities in terms of physiological and anatomical between human and pig. The decision of choosing pig's ear skin in the experiment was because it represented a suitable model for human skin according to Jacobi studied in 2005⁸. Besides that, human skin has a disadvantage of becoming contracted during excision. This causes the hair follicles to be permanently blocked the contracted elastic fibers⁹. Thus, pig's ear skin can be described as superior to excised human skin in follicular penetration studies. Acne vulgaris is one of the most common skin disorders in 80 % of most adolescents, though it can continue to occur in adulthood especially in women due to hormonal imbalance during menstrual cycle. Comedones, inflamed papules, pustules and nodules can be observed in the lesion of acne. Acne is mostly present in the highest number of pilosebaceous glands area such as the face, chest, and back¹⁰.

Figure 3: Acne Pathogenesis

Pilosebaceous unit which composed of hair follicle, hair shaft and the sebaceous gland is the main target location for acne lesions to occur. The first process for the development of acne is the formation of the micromedo. Micromedo acts as a precursor for the occurrence of the acne lead to comedone formation. Accumulation of keratinocytes due to disturbed desquamation at the follicular fundibulum leads to the formation of micromedo. Occluded follicle contained adherent keratinocytes and androgen-driven activation of sebum secretion can also lead to the formation of micromedo. In addition, colonization of *Propionibacterium acne* (*P. acne*) in the pilosebaceous unit causes inflammatory acne lesions¹¹. The use of antimicrobial therapy or antibiotics in the treatment of acne has started since 1930s and 1940s. Although antibiotics have been a part of mainstay treatment for a long time, acne experts recommend it to act as an adjunctive therapy instead of primary in the role of acne treatment¹¹. The mechanism on how topical antibiotics help to improve acne has not been clearly defined. They are said to act directly on *P. acne* colonization and thus produces pro-inflammatory actions on comedogenesis. The most commonly used topical antibiotics on acne are clindamycin and erythromycin¹². However, topical gentamicin sulfate can also be used to treat

secondary skin infections including pustular acne¹³. Gentamicin belongs to the aminoglycoside family of antibiotics. It is hydrophilic in nature and covers a broad spectrum of bacteria. It has a low molecular weight of approximately 500 g/mol. It has an anti-inflammatory effect by working through inhibition of protein synthesis and messenger ribonucleic acid translation, thereby stopping the growth of certain bacteria¹⁴. Gentamicin sulfate cream USP 0.1 % is a wide spectrum topical antibiotic preparation and is used to treat primary and secondary skin infections including pustular acne. The topical preparation of gentamicin sulfate helps to control the infection in the treatment of underlying skin disease. Bacteria that is sensitive to the action of gentamicin sulfate including sensitive strains of *Streptococci*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Aerobacteraerogenes*, *Escherichia coli*, *Proteus vulgaris* and *Klebsiella pneumonia*¹⁵.

Figure 4: Structure of Gentamicin

Hence, the objective of the present study was to confirm permeation pathway of gentamicin through follicular route. The further, aim was to compare drug concentration of gentamicin between plugged and non-plugged skin.

MATERIALS AND METHODS

Materials

Pig's ear skins were purchased from Pigs farm situated in Kuala Selangor, Selangor Darul Ehsan, Malaysia. The study was performed on two pig ears from freshly slaughtered pigs. Model penetrant of gentamicin sulfate cream 0.1 % USP was obtained from HOVID BHD, (Jln Tunku Abd. Rahman, Ipoh, Malaysia). Silicone grease was obtained from Synco Chemical Co., Ltd., (Bohemia, NY, USA). Nile red coloring agent was purchased from PPB Group Berhad, (Kuala Lumpur, Malaysia). Potassium dihydrogen phosphate (KH_2PO_4), Sodium chloride (NaCl), Disodium phosphate (Na_2HPO_4), Hydrochloric acid (HCl), Methanol (CH_3OH) was obtained from Sigma Aldrich Co., Ltd (St. Louis, MO, USA). Toothpick stick was purchased from Kim Ban Marketing, Shah Alam, Selangor, Malaysia. Eppendorf tubes were obtained from Sigma Aldrich Co., Ltd., St. Louis, MO, USA.

Equipments

Franz vertical glass diffusion cell was purchased from Perme Gear Inc., Hellertown, USA. UV Spectrophotometer was purchased from Thermo Fischer Scientific, Inc., Waltham, MA, USA. Sonicator was obtained from Restek, Bellefonte, PA, USA. Water bath TC-502D-230 was obtained from Brook field Engineering Laboratories, Inc. (Middleborough, MA, U. S. A). pH Meter was purchased from Hanna Instrument Ltd., Woonsocket, RI, USA. Analytical balance GR-200 was obtained from A and D Company Ltd., Toshimaku, Tokyo, Japan. Shaver series 500 was obtained from Koninklijke

Philips Electronics N.V., Kuala Lumpur, Malaysia. Magnifying glass was obtained from Lik Soon Sdn Bhd., Kuala Lumpur, Malaysia.

Methods

Figure 5: Methodology for comparison of gentamicin skin permeation through skin

Preparation of Excised Pig Ear Skin

The pig ears were cleaned with distilled water and washed out of minerals for 2 hours before the experiments. Pig ear skin hair was then removed to obtain a clean shaved skin. The fat under the pig ear skin was also removed and only the stratum corneum and the epidermis/dermis were remained.

Preparation of Plugging Agent

0.2 g of silicone grease was mixed well with 0.25 mg of Nile red agent to obtain a red hair follicle plugging agent.

Preparation of Plugged Pig Ear Skin

Polypropylene stick with diameter of 0.5 mm was used to cover or plug the hair follicles of the shaved pig ear skin with the plugging agent prepared. This step required the aid of magnifying glass.

Preparation of Phosphate Buffer Saline (PBS)

0.9073 g of potassium phosphate (KH_2PO_4) and 1.1688 g of sodium chloride (NaCl) was weighed and mixed up to 200 ml of distilled water. Next, 9.5504 g of sodium phosphate (Na_2HPO_4) and 4.6752 g of sodium chloride (NaCl) was prepared and mixed up to 800 ml of distilled water. Then, 200 ml mixture was added to the 800 ml mixture and stirred well to get 1 liter of PBS pH 7.4.

Multi-Station Vertical Franz Diffusion Cell System

A circular piece of the pig ear skin was sandwiched securely between the two halves of the Franz diffusion cells with the stratum corneum side facing the donor chamber. The top donor compartment was separated from the bottom receptor compartment (filled with a liquid) by a piece of excised pig ear skin. Gentamicin sulfate cream

was applied on the pig ear skin in the donor chamber. The donor chamber was covered with a paraffin film. Each of the receiver chambers were filled with 6 ml of PBS (pH 7.4) and continuously stirred using a magnetic stirrer and thermo stated at $32 \pm 0.2^\circ\text{C}$ throughout the experiment. The steps were performed for both plugged and non-plugged pig's ear skin twice for drug concentration comparison. Vertical Franz diffusion cell system is used as an *ex vivo* passive diffusion experiments through excised skin. This method is standardized through Test Guidelines for *in-vitro* Assessment of Dermal Absorption and Percutaneous Penetration of Cosmetic Ingredients invented by Diembeck in 1999¹⁶. Vertical Franz cell method allows diffusion and storage of penetrant molecules in skin layers to be assessed quantitatively at a macroscopic level.

Collection of Samples

The diffusion cell system was running for 24 hours. 1 ml of the sample was drawn out for each specified time interval. First sample was drawn out at 0 minute prior to topical gentamicin application. The next samples were taken for every half an hour after the topical gentamicin application started from 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5 up to 8 hours. The cell system was then let to run for 10 hours without any collection of samples. The next samples were taken at 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5 and 24 hours. The receiver chamber was top up with 1 ml of PBS every time the samples were collected. All of the steps were carried out for both plugged and unplugged skin.

Preparation Stock Solution for Standard Curve in UV Spectrophotometer

3.3 ml of gentamicin 0.3 % was diluted with 30 ml methanol with concentration of 1000 mcg/ml for stock solution. Serial dilution with known concentration was then performed from the stock solution prepared.

- **100 µg/ml**
1 ml of the stock solution was diluted up to 10 ml with PBS
- **80 µg/ml**
0.8 ml of the stock solution was diluted up to 10 ml with PBS
- **60 µg/ml**
0.6 ml of the stock solution was diluted up to 10 ml with PBS
- **40 µg/ml**
0.4 ml of the stock solution was diluted up to 10 ml with PBS
- **20 µg/ml**
0.2 ml of the stock solution was diluted up to 10 ml with PBS
- **10 µg/ml**
0.1 ml of the stock solution was diluted up to 10 ml with PBS
- **5 µg/ml**
0.05 ml of the stock solution was diluted up to 10 ml with PBS

Absorbance against known concentration (mcg/ml) standard curve for gentamicin was plotted.

Analysis of Samples by UV Spectroscopy

The absorbance measured from the unknown concentration of the samples for each time intervals were recorded and compared with known concentration of standard curve of gentamicin to obtain concentration of the samples. From the concentration obtained, cumulative amount of permeation pathway for gentamicin through plugged and non-plugged skin was calculated.

RESULTS AND DISCUSSION

Calibration Standard Curve for Gentamicin

Figure 6: Calibration curve for gentamicin in phosphate buffer solution (pH 7.4)

The graph was plotted based on the known concentration obtained from the serial dilution of gentamicin. Based from the graph, equation $y = 0.0003x + 0.0029$ was obtained along with the R^2 value. From the equation, concentration for the plugged and non-plugged skin samples can be calculated from absorbance measured by UV Spectrophotometer.

Determination Concentration of Gentamicin through Non-Plugged and Plugged Skin

In order to find concentration of the samples for each time intervals from the absorbance, the x-value should be defined from the equation, $y = mx + c$.

$$y = mx + c$$

$$y = 0.0003x + 0.0029$$

$$x = (y - 0.0029) / 0.0003,$$

Where; y = absorbance, x = concentration

Table 1: Concentration (y-value) for non-plugged and plugged skin samples for each time intervals obtained from calculated equation using absorbance (x-value)

Time (Hour)	Non-plugged skin		Plugged skin	
	Absorbance (y-value)	Concentration (x-value)	Absorbance (y-value)	Concentration (x-value)
0	0	0	0	0
0.5	0.18	583.67	0.01	20.33
1.0	0.20	643.67	0.04	107.00
1.5	0.24	773.67	0.09	280.33
2.0	0.26	853.67	0.14	443.67
2.5	0.27	880.33	0.16	513.67
3.0	0.27	887.00	0.17	550.33
3.5	0.27	893.67	0.17	560.33
4.0	0.25	823.67	0.17	543.67
4.5	0.23	750.33	0.16	530.33
5.0	0.24	793.67	0.16	523.67
5.5	0.25	813.67	0.16	517.00
6.0	0.23	770.33	0.16	507.00
6.5	0.21	703.67	0.15	490.33
7.0	0.23	740.33	0.15	483.67
7.5	0.19	617.00	0.15	473.67
8.0	0.18	603.67	0.14	460.33
18.0	0.15	487.00	0.10	317.00
18.5	0.14	467.00	0.09	300.33
19.0	0.14	440.33	0.09	280.33
19.5	0.12	400.33	0.08	270.33
20.0	0.13	427.00	0.08	260.33
20.5	0.13	423.67	0.08	253.67
21.0	0.13	410.33	0.07	237.00
21.5	0.12	380.33	0.08	240.33
22.0	0.10	337.00	0.07	227.00
22.5	0.10	330.33	0.06	200.33
23.0	0.10	323.67	0.06	193.67
23.5	0.09	303.67	0.06	193.67
24.0	0.09	280.33	0.06	187.00

Figure 7: Concentration over time profile for gentamicin permeation pathway through non-plugged skin. Half life of gentamicin is 2-4 hours which explained the decreasing in trend for gentamicin concentrations in non-plugged skin after 4 hours. From the graph plotted, equation $y = -17.731x + 772.97$ was obtained, in which $m = -17.731$ and $c = 772.97$

Figure 8: Concentration over time profile for gentamicin permeation pathway through plugged skin. Half life of gentamicin is 2-4 hours which explained the decreasing in trend for gentamicin concentrations in plugged skin after 4 hours. From the graph plotted, equation $y = -9.7825x + 465.69$ was obtained, in which $m = -9.7825$ and $c = 465.69$

Figure 9: Cumulative amounts of permeation gentamicin/available permeation area ($\mu\text{g}/\text{cm}^2$) for plugged and non-plugged skin. The results are significant differences ($P < 0.05$) between concentrations of plugged and non-plugged skin

Figure 9 shows the comparison between cumulative amount of permeation gentamicin/available permeation area ($\mu\text{g}/\text{cm}^2$) in plugged and non-plugged skin. Based on the results, the rate of gentamicin permeation into the skin was higher when the hair follicles orifices were open as compared to the plugged skin. This

indicates high amount of gentamicin was permeated across the skin for non-plugged skin and lower amount of gentamicin permeated through plugged skin. The concentration for the gentamicin was found higher after 8 hours which is on the 18 hours as the samples were left for 10 hours before next samples collection.

Determination Comparison Cumulative Amount of Permeation Gentamicin over Available Permeation Area through Plugged and Non-Plugged Skin**Table 2: Cumulative amount of permeation gentamicin/available permeation area ($\mu\text{g}/\text{cm}^2$) for plugged and non-plugged skin**

Time (Hour)	Cumulative amount of permeation drug/available permeation area ($\mu\text{g}/\text{cm}^2$)	
	Non-plugged skin	Plugged skin
0	0	0
0.5	1648.79	57.43
1.0	2148.03	313.75
1.5	2878.92	863.83
2.0	3542.01	1483.62
2.5	4099.62	1932.02
3.0	4615.82	2325.79
3.5	5135.80	2664.96
4.0	5442.95	2934.47
4.5	5701.13	3203.94
5.0	6247.47	3493.23
5.5	6752.37	3763.46
6.0	7089.64	4027.31
6.5	7336.55	4266.66
7.0	7837.67	4524.86
7.5	7907.54	4769.88
8.0	8218.47	4999.80
18.0	8229.95	4854.99
18.5	8883.81	5273.44
19.0	8637.10	5100.18
19.5	8772.88	5230.31
20.0	9074.40	5354.79
20.5	9306.23	5483.05
21.0	9507.91	5579.28
21.5	9654.99	5722.58
22.0	9747.46	5820.71
22.5	9919.02	5873.62
23.0	10086.83	5967.98
23.5	10213.20	6077.40
24.0	10318.83	6167.98

Calculation for Skin Permeation Parameters

P = Apparent permeability coefficient

P of non-plugged skin = $(-17.731 \div 1000 \text{ mcg/ml})$

P of plugged skin = $(-9.7825 \div 1000 \text{ mcg/ml})$

Skin permeation-decreasing ratio (%) = $[(P \text{ of non-plugged} - P \text{ of plugged}) \div P \text{ of non-plugged}] \times 100$
 $= [(-17.731 - (-9.7825)) \div (-17.731)] \times 100$
 $= 44.83 \%$

DISCUSSION

The absorption of topically applied gentamicin on the skin with and without the influence of hair follicles was studied. In non-plugged skin which the hair follicle orifices were open, there was a significant high amount of gentamicin was detected. As compared to a plugged skin in which the hair follicle orifices were artificially blocked, gentamicin can only penetrate through interfollicular epidermis and possible through sweat glands. Thus, the maximum concentration for the drug permeated through non-plugged skin was generally lower than in the plugged skin. From the results obtained, it clearly showed that the follicular route is a significant access route for chemical compounds which is important in development and optimization of topically applied drugs and cosmetics. The two-tailed P value result is less than 0.05 ($P < 0.05$) between concentrations of plugged and non-plugged skin. By conventional criteria, this difference is considered to be statistically significant. Percentage differences between gentamicin permeated through plugged and non-plugged skin was obtained from skin-

permeation decreasing ratio. The calculated percentage showed 44.83 % differences which was slightly significant. Corresponding investigations have been performed with different type of compound. To determine the extent of caffeine penetration via hair follicles in 2006 using the nail varnish method called the Closed Follicle Technique or FTC showed that the follicles contribute about 50 % of the total caffeine permeation¹⁷. Trauer also proved that increase in density of hair follicle resulted in delayed skin penetration *in-vitro* in the condition of the evacuation by the blood and lymph system is absent¹⁷. In addition, the role of hair follicles in the percutaneous absorption of caffeine that faster absorption of caffeine was obtained when the follicle orifices were open¹⁸. The results revealed that the caffeine were detectable within 5 minutes when the hair follicle orifices remained open. When the follicular orifices were closed, penetration of caffeine into the skin took longer time which was 20 minutes and lower blood caffeine concentration was measured. Hence, the study performed shows clear differences between intercellular and follicular drug permeation pathway *in vivo*¹⁹. This proves that follicular route is highly significant for the permeation of drugs. A hydrophilic drug is unable to penetrate the skin easily as it cannot enter the hydrophobic layer of stratum corneum³. Thus, skin appendages including hair follicles route gained renewed interest as it shown to be the leading pathway for hydrophilic drugs²⁰. There are several targets of cosmetic and pharmaceutical interests on structure of the follicle. Follicles are located deep inside the skin where the SC is thinner and the vascularization is denser²¹. Hence, topically applied compounds generally permeate into the central circulatory systems when they reach the cutaneous vasculature which results in rapid dilution⁵. The topically applied compound is then dispersed within the entire circulatory system²². This shows that follicular route is highly significant for the efficacy of systemic drugs. Another potential

target for drug delivery against acne is the sebaceous gland which is associated with the hair follicles. Besides that, reservoir of keratinocytes stem cells of follicle which is bulge region deeper in the follicles is also becoming the huge interest target for drug delivery²³. Infundibulum of the hair follicles acts as a long term reservoir and thus is able to delay penetration of nanoparticles. Hence, infundibulum plays a main role in penetration processes²⁴. Lademann stated in his studies in 2006 that it has been measured the hair follicles possess a ten-fold longer storage period compared to inside the SC²⁵. In addition, the hair follicles act as a good long-term reservoir for topically applied substances up to 10 days. This is because the depletion of particles on the hair follicles occurs only through slow processes of sebum production and hair growth²⁶. Limitation of follicular penetration is there might be a possibility that the follicle orifice is blocked by a plug. This limitation however, can be overcome by peeling the skin or by surface skin stripping with cyanoacrylate tapes²⁷. Besides that, the analysis instrumentation used for the study can be improved by using a new surface ionization mass spectrometry (SI/MS) technique, which is able to show clear differences between interfollicular and follicular penetration of a topically applied substance as compared to UV Spectrometry¹⁹. The calculated percentage showed 44.83 % differences between gentamicin permeation through plugged and non-plugged skin which was slightly significant. Further study is suggested to perform by using raw materials of gentamicin instead of gentamicin cream used in the present study. If the calculated percentage differences of gentamicin permeation pathway are very significant approaching nearly to 100 %. New formulation for topically-applied gentamicin can be formulated. Several studies have shown an increased follicular penetration by particles smaller than 300 nm. Nanoparticles penetrate hair follicles very efficiently²⁸. This can improve the efficacy of topically-applied compounds on hair-follicles associated disease such as acne vulgaris^{29,30}.

CONCLUSION

The findings of this study demonstrated that hair follicles were significant access penetration route for gentamicin. The permeation of gentamicin through non-plugged skin was higher as compared to the plugged skin. The present study showed statistically significant results with p-value < 0.05. The important role of hair follicles as an ideal long term reservoir and effective site of penetration has been validated in numerous studies involving animal and human skins. The present study was limited to only one compound, gentamicin. More research and investigation can be done to evaluate the role of hair follicles in drug penetration with different type of compounds which is significant in development and optimization of topically applied drugs and cosmetics. This study is beneficial for the further development of follicular targeting mechanism in clinical applications.

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Conflict of Interests

The authors declare that they have no competing interests.

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