In vitro skin permeation of sinigrin from its phytosome complex

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Abstract

Objectives: Sinigrin is a major glucosinolate present in plants of the Brassicaceae family. Recently sinigrin and its phytosome formulations have been investigated for its wound healing actions, by our research group. The aim of this study was to demonstrate sinigrin drug release from its phytosome complex and also to determine if the phytosome complex enhances the delivery of sinigrin into the skin when compared to free sinigrin.

Methods: In vitro Franz cell diffusion studies were performed on human abdominal skin. The morphology of the phytosome complex was examined by transmission electron microscopy. The in vitro drug release was determined by using dialysis sacks.

Key findings: The in vitro drug release indicated a controlled and sustained release of sinigrin from the phytosome complex. Tape stripping results showed that the sinigrin-phytosome complex (0.5155 µg/ml) statistically significantly enhanced the delivery of sinigrin into the stratum corneum-epidermis when compared to the free sinigrin (0.0730 µg/ml).

Conclusions: These results suggested the possibility of utilizing sinigrin-phytosome complex, to optimally deliver sinigrin to the skin which can be further used for various skin related diseases including wound healing.

Keywords: Sinigrin, Phytosome, Sinigrin-phytosome complex, Skin permeation, Tape stripping
1. Introduction

The efficacy of employing the skin as the port of drug administration to the human body has been known for many years. Since the skin constitutes an excellent natural and protective barrier against the delivery of the therapeutic agents; topical and transdermal drug delivery is very onerous. The skin is the largest organ in human body with a surface area of 1.8-2.0m² comprising of three main layers; the epidermis, dermis and hypodermis (subcutaneous layer). The epidermis contains an uppermost layer of cells called the stratum corneum, which is the main hindrance of the skin and consists of corneocytes surrounded by lipid regions. The viable epidermis is approximately 100–150 µm thick and consist of multiple layers of keratinocytes and many other types of cells. The stratum corneum lipids play a crucial part in maintaining and structuring the lipid barrier which supplies protection against external insults and water loss through the skin and is the cause of selective permeability.

The penetration of drugs through the skin includes the diffusion through the entire epidermis and the skin appendages (hair follicles and sweat glands). Topical drug delivery provides advantages like controlled and sustained drug release, less fluctuations in plasma drug levels, avoidance of first-pass metabolism, better patient compliance and enhanced local (dermal) or systemic (transdermal) effects.

Several methods have been investigated to enhance the permeation of therapeutic agents through the skin and one such method is using a transdermal nanocarrier delivery system.

Many vehicle systems have been suggested to transport drugs through the skin, facilitating drug retention and allowing a sustained release. One such vehicle, used recently for transdermal drug delivery, is known as phytosomes or herbosomes.

Phytosomes are a vesicular drug delivery system in which phytoconstituents are surrounded, bound and linked with a phospholipid molecule. Phytosomes have been renowned for enhanced permeation of therapeutic agents through the skin during transdermal and dermal delivery and when used as functional cosmetics, protect the skin against exogenous or endogenous hazards in normal conditions as well as during a stressful environment, thus
providing synergistic effects. Since phosphatidylcholine, being an essential part of the cell membrane, is used in preparing phytosome formulations it acts as a carrier and also nourishes the skin. The transition of the phytoconstituents linked to phospholipids takes place through interaction with the cutaneous structure of the skin, which helps in the release of the bioactives. Phytosome complexes augment the rate of absorption of the phytoconstituents through the skin to regulate the physiology of skin composition (without damaging the epidermis), which highlights the potential utilization of phytosomes in cosmetics as well as for systemic function via the skin.

Sinigrin is a type of glucosinolate mainly found in the seeds of Brassica nigra and other members of the Brassicaceae family, such as Brussels sprouts, broccoli and cabbage. Figure 1 represents chemical structure of sinigrin. Glucosinolates appear as secondary metabolites that are characteristic of plants of the Brassicaceae family. It is well known that glucosinolates are broken down enzymatically by myrosinase, mainly to isothiocyanates, cyanides and thiocyanates which are the main known bioactives and are generally attributed for its therapeutic benefits. In a recent study conducted in our laboratory, we have demonstrated the potential wound healing activity of sinigrin and its phytosome formulations. The aforementioned steered our interest in examining the in vitro topical delivery of sinigrin and its phytosome complex. To date, there is no information concerning the topical delivery of sinigrin and its phytosome complexes, making this research work novel. We also endeavoured to demonstrate the in vitro drug release of the free sinigrin and sinigrin-phytosome complexes by deploying a dialysis bag in our current study. Figure 2 depicts the graphical image of the in vitro skin permeation of the sinigrin from its phytosome complex.

2. Materials and methods

2.1 Materials

Sodium hydroxide (NaOH) and potassium dihydrogen orthophosphate (KH₂PO₄) was purchased from Merck, USA, sinigrin (purity≥98%) was purchased from Santa Cruz
Biotechnology, Germany. L-α- phosphatidylcholine hydrogenated (soya bean) (≥99%) was procured from Sigma- Aldrich, USA and solvents, such as dichloromethane (DCM) and n-hexane, were obtained from Sigma Aldrich, USA.

2.2 Preparation of sinigrin-phytosome complex

Sinigrin-phytosome complexes were prepared as per the previously described method\textsuperscript{13}. Phosphatidylcholine (PC) was combined with sinigrin, in a ratio of 1:1 (w/w), with 5ml of DCM and stirred well while evaporating the DCM. When the DCM was completely removed, 5ml of n-hexane was added to the thin film, mixed well and then the solvent was allowed to completely evaporate. After the n-hexane was completely evaporated, the thin film was hydrated and probe sonicated for about few minutes (Hielscher UP 200ST ultrasonic device, South Africa), to get the desired size phytosome vesicles.

2.3 Transmission electron microscopy

The samples were diluted with distilled water, and then a drop of the phospholipid vesicles was placed onto a copper grid, leaving a thin liquid film. The films on the grid were then stained with 1% osmium tetroxide and excess staining solution was removed using filter paper, this was then allowed to dry for 15 mins. The stained films were observed and photographed using transmission electron microscopy (TEM) (model FEI Tecnai\textsuperscript{TM} G2, USA), at an accelerating voltage of 120 kV, with images captured by a GATAN bottom mount camera using digital micrographics.

2.4 In vitro drug release studies

The in vitro drug release studies were accomplished using the dialysis method at room temperature. Dialysis sacks (Sigma Aldrich, USA), with a molecular weight cut-off ranging from 12,000-14,000, were utilized during these experiments as the donor compartment. Details for this method have been provided in supplementary data.
2.5 Preparation of phosphate buffer solution

The PBS (pH 7.4) used as the receptor solution during the *in vitro* permeation studies was prepared according to the British Pharmacopoeia (British Pharmacopoeia, 2015)\(^\text{14}\). Details for this method have been provided in supplementary data.

2.6 High performance liquid chromatography (HPLC) analysis of sinigrin

The HPLC method for the analysis of sinigrin was developed and validated according to ICH guidelines\(^\text{15}\). The HPLC (Agilent 1100 series, Agilent, Palo Alto, USA) system consists of an auto-sampler, variable wavelength detector (VWD) and an isocratic pump. A chromatographic column (Venusil XBP C\(_{18}\), 5\(\mu\)m, 150x 4.6mm, Agela Technologies, Newark, USA) was used for analytic separation. The mobile phase comprises of a mixture of tetrabutyl ammonium bisulfate (TBASO\(_4\)) and acetonitrile (80:20, v/v). TBASO\(_4\) was prepared by dissolving it in deionized water (1.7g/l) and adjusting the pH to 7.3 \(\pm\) 0.2. The flow rate was kept at 1.0 ml/min, with a sample injection of 50\(\mu\)l volume and UV detection at 228 nm. Fresh standard solutions of sinigrin dissolved in distilled water were prepared before each study and injected into the HPLC to obtain calibration curves ranging from 0.60 to 12.00\(\mu\)g/ml in concentration. Limit of detection (LOD) was 0.015\(\mu\)g/ml and limit of quantification (LOQ) was 0.07\(\mu\)g/ml.

2.7. *In vitro* skin permeation and tape stripping

2.7.1 Skin preparation

Permeation studies were performed using abdominal skin obtained from Caucasian female patients after abdominal plastic surgery\(^\text{16}\). Prior to the surgery, informed consent was obtained from all the patients, with donor identities kept confidential. Within 24 hrs after the surgery, the skin was placed in plastic bags and frozen at -20°C until used. The research ethics committee of the North-West University (Pochefstroom Campus) authorized ethical approval for the procurement and use of the donated skin under reference number NWU-00114-11-A5 (2011-08-25). Details for this method have been provided in supplementary data.
2.7.2 Skin diffusion studies

Vertical Franz diffusion cells with an active diffusion area of 1.13 cm$^2$ and receptor volume of 2 ml were used for the \textit{in vitro} permeation studies. To maintain constant stirring of the PBS (pH 7.4) receptor solution, a small magnetic stirrer bar was placed in the receptor compartment of each Franz cell. Thereafter, the dermatomed skin was placed between the receptor and donor compartments with the stratum corneum facing the donor compartment. The two compartments were subsequently sealed with Dow Corning$^\text{®}$ high vacuum grease and clamped together using a horseshoe clamp to prevent leakage. The donor compartments were filled with 1ml of the donor solution, preheated to 32 °C in a water bath to match normal human skin temperature. Parafilm$^\text{®}$ was used to cover the donor compartments to prevent evaporation of the donor solutions during the diffusion. The receptor compartments were filled with 2 ml PBS (pH 7.4), preheated to 37 °C, taking care that no air bubbles formed underneath the skin. The receptor compartments were submerged in water within a water bath set at 37°C to achieve a skin surface temperature of 32°C $^{16}$. After 12 hrs, the entire volume of the receptor compartment was withdrawn for HPLC analyzed. Details for this method have been provided in supplementary data.

2.7.3 Tape stripping

Tape stripping is a technique used to investigate the amount of active ingredient which penetrated the outer layers of the skin after application of a formulation$^{17}$. During this study tape stripping was performed according to a method described previously$^{18,19,20}$. After completion of the 12 hrs permeation studies, the Franz cells were taken apart and the skin of each cell pinned to Parafilm$^\text{®}$ supported by a steady surface. The skin area exposed during the diffusion was distinctly indented by the Franz cells. Some of the remaining donor solution present on the surface of the skin was removed using dry paper towel. 3M Scotch$^\text{®}$ Magic™ tape was cut into pieces (16) large enough to cover the diffusion area. The first tape strip applied on the skin was discarded as part of the cleaning process as it is may have been contaminated with sinigrin adhering to the surface of the skin. In order to remove the
stratum corneum, the following 15 tape strips applied on the skin were placed in a vial filled with PBS (pH 7.4) and stored at 4 °C overnight (referred to as stratum corneum-epidermis\textsuperscript{18,19,20}. After tape stripping, the left over skin (referred to epidermis-dermis) was cut into smaller pieces and placed in a vial containing PBS (pH 7.4) for storage at 4 °C overnight. Samples were withdrawn from each of the vials containing the tape strips and the left over skin, filtered with 0.45 µm syringe filters and analyzed by HPLC.

2.8 Statistical data analysis

For the \textit{in vitro} permeation studies, the mean amount per area (µg/cm\textsuperscript{2}) of sinigrin which diffused through the skin after 12 hrs was calculated. The concentration (µg/ml) sinigrin delivered into the skin layers (stratum corneum-epidermis and epidermis-dermis) was determined from the results obtained from the tape stripping studies. Statistica data analysis software, version 12 \textsuperscript{21} (StatSoft, Inc., 2015) was used to examine the data obtained from the skin diffusion and tape stripping studies.

Firstly, data was analyzed by descriptive statistics, which involved the calculation of the mean (with standard deviation) and median (measure of central location) values\textsuperscript{22,23}.

Secondly, the data was analyzed by comparative statistics, namely the independent sample \textit{t}-test, to determine whether there was a statistical significant difference\textsuperscript{22} between the control study (sinigrin in water) and the phytosome-sinigrin complex. Statistical significant differences were indicated when a p-value < 0.05 was observed.

\textit{In vitro} drug release study data were analyzed using GraphPad Prism™ Software (version 5.01, USA). Statistical significance was determined with two-way ANOVA statistics. Data are presented as mean ± standard deviation (SD) and significance was set at p < 0.05.

3. Results and Discussion

3.1 Transmission electron microscopy

The sinigrin-phytosome complex was characterized with respect to size, zeta-potential, structural morphology and complex formation efficiency previously by our research group\textsuperscript{13}.
TEM, illustrated in Figure 3, provided the evidence of vesicle formation, displaying a uniform, spherical shape with smooth outer surfaces and with little or no signs of agglomeration.

### 3.2 In vitro drug release

The *in vitro* drug release profiles of the free sinigrin and sinigrin-phytosome complex are shown in Figure 4. The result revealed that the free sinigrin was completely released within 2 hrs, while the sinigrin-phytosome complex released 100% of sinigrin during a time span of 8 hrs. After 2 hrs, only 45.5% of sinigrin was released from the phytosome complex. The data also demonstrated that the sinigrin release from phytosome complex was in a controlled and sustained manner. The steady slow release of sinigrin from both phytosome complex indicated that homogeneous phytosome complex formation of sinigrin had occurred. A similar study has been conducted where Ashwagandha phytosome complex and a conventional formulation were subjected to an *in vitro* drug release study. It was found that the phytosome complex had the highest cumulative percentage drug release compared to the conventional formulation. Sumathi and their research group formulated phytosomes containing methanolic extracts of *Nymphaea nouchali* and *trichosanthes dioica* and also demonstrated the *in vitro* drug release. Their findings proposed that the phytosome vesicles containing *Nymphaea nouchali* and *Trichosanthes dioica* attained the maximum percentage of extract release with longer duration of time, with enhanced *in vitro* release characteristics in compared to the crude extracts.

### 3.3 In vitro permeation studies and tape stripping

No sinigrin was observed in the receptor phase when sinigrin-phytosome complexes were applied to the skin for 12 hrs (Figure 5). Nevertheless, the mean amount per area of sinigrin which diffused through the skin after 12 hrs, during the control study (free sinigrin), was $2.102 \pm 1.547 \mu g/cm^2$. This may be due to the quick release of sinigrin (within 2 hrs) from the free sinigrin solutions seen in the *in vitro* release studies. However, as the data set contains extreme values, the median value is a more suitable measure of the central location than the mean value, consequently the median amount per area sinigrin that permeated through the
skin after 12 hrs is more representative of the true value, and was found to be 1.664 µg/cm² for the control study.

Successful research has been recognized indicating that the phytosome technology has the therapeutic activities to be used transdermally for increasing bioavailability of phytoconstituents. A study by Das and Kalita, showed the skin permeability, after 24 hours (over rat abdominal skin), of the flavonoid rutin, was enhanced when phytosome complex was formed (compared to the uncomplexed rutin). After 24 hrs, the rutin content found in the skin, via extraction, was also higher with the rutin-phytosome complexes than the pure rutin. This indicated the rutin phytosomes were able to penetrate the stratum corneum better than the free rutin. This higher amount of retention in the skin suggests that phytosomes are able to deliver the rutin over an extended period of time. Another similar study showed that resveratrol phytosomes also had higher skin permeation over 24 hrs and retention at the dermal sites than the uncomplexed resveratrol. The skin permeation of curcumin with the phytosome curcumin was studied and it was found that complexed curcumin showed 60% greater permeation of curcumin through rat skin, suggesting that phytosome complex had better transdermal penetration than curcumin alone.

The main aim of the current study was to determine whether the phytosome complex enhanced the delivery of sinigrin into the skin layers by means of performing tape stripping on the skin after the 12 hrs diffusion studies. Direct delivery to the wound site is desirable for therapeutic agents, such as sinigrin, which are involved in the wound healing process. Tape stripping results showed neither the control nor the sinigrin phytosome complex delivered sinigrin into the epidermis-dermis. However, as can be seen in Figure 5, the sinigrin phytosome delivered a significantly (p < 0.001) higher mean concentration sinigrin (0.507 ± 0.041 µg/ml) into the stratum corneum-epidermis than the control solution (0.096 ± 0.116 µg/ml). However, as mentioned before, it is proposed to use the median concentration values as these values are not influenced by a distortion in the data range. Thus, when comparing the median concentration (Figure 5) of sinigrin delivered to the stratum corneum-epidermis, it was determined that the phytosome-sinigrin complex and the control solution
delivered sinigrin into this skin layer at concentrations of 0.5155 µg/ml and 0.0730 µg/ml, respectively. The higher retention of sinigrin into this skin layer by the phytosome-sinigrin complex may be explained by its gradual release of sinigrin from the phytosome over time (as seen in Figure 4). Another explanation for the excellent delivery of sinigrin by phytosomes into the stratum corneum-epidermis skin layer involves the formation of lipid-compatible molecular complexes when the water soluble sinigrin are bound to phospholipids. These results suggest the control solution caused the sinigrin to mostly diffuse through the skin layers, thereby causing only a small concentration of sinigrin to accumulate in the stratum corneum-epidermis layer. Additionally, it is proposed that phytosome complexes can potentially be used to directly deliver a higher concentration of the phytoactive sinigrin, to the wound site, which is confirmed by our recent study.

4. Conclusion

Sinigrin-phytosome complex exhibited no diffusion through the skin after 12 hrs, however, results showed the phytosome complex were able to deliver a statistically significantly higher concentration of sinigrin into the stratum corneum-epidermis when compared to the control (free sinigrin). Therefore, it suggests that sinigrin phytosomes can be used to improve the delivery of this phytocconstituent to the target site for wound healing, which is congruent with our recent investigation. It was also noteworthy that sinigrin was released in a slow and controlled manner from the phytosome formulation. Furthermore, sinigrin-phytosome complex has the potential to be used for various skin-related diseases and cosmetic products. Further studies include investigation of the mechanism of action, permeation through compromised skin, determination of the physiochemical properties of the complex.

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Conflict of interest

The authors declare no conflict of interest.

Disclaimer

Any opinions, findings, conclusions, or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.
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**Figure legends**

**Figure 1.** Chemical structure of sinigrin

**Figure 2.** Illustration of graphical image of the *in vitro* skin permeation of sinigrin phytosome complex.

**Figure 3.** TEM pictures of sinigrin-phytosome complexes

**Figure 4.** *In vitro* release of free sinigrin and sinigrin-phytosome complex. Each value represents the mean and SD of three experiments.

**Figure 5.** Box plots to illustrate the concentration (µg/ml) sinigrin present in the stratum corneum-epidermis for the sinigrin-phytosome complex and free sinigrin. The mean and median concentration values are shown as squares and plus signs, respectively.
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Supplementary Data

2.4 In vitro drug release studies

The in vitro drug release studies were accomplished using the dialysis method at room temperature. Dialysis sacks (Sigma Aldrich, USA), with a molecular weight cut-off ranging from 12,000-14,000, were utilized during these experiments as the donor compartment. The dialysis bags were soaked in double-distilled water for 2 hrs before use, and then 4ml of freshly prepared sinigrin-phytosome dispersion and sinigrin was poured respectively into each bag with the two ends fixed by clamps. The bags were placed in a conical flask filled with a receptor medium consisting 40ml phosphate buffer (PBS pH 7.4, PBS tablets was dissolved in water). The conical flasks were placed on a magnetic stirrer plate at 37°C at 100 rpm. From each sample, 2ml were extracted at selected time intervals and the volume was replaced with same amount of fresh PBS following each sampling. Samples were filtered through 0.45 μm membrane filters and assayed by HPLC.

2.5 Preparation of phosphate buffer solution

The PBS (pH 7.4) used as the receptor solution during the in vitro permeation studies was prepared according to the British Pharmacopoeia (British Pharmacopoeia, 2015), incorporating 250.0ml of a 0.2M KH₂PO₄ solution to 393.4 ml of a 0.1M NaOH solution, where-after the pH of the solution was adjusted to pH 7.4 using phosphoric acid (H₃PO₄).

2.7.1 Skin preparation

Permeation studies were performed using abdominal skin obtained from Caucasian female patients after abdominal plastic surgery. Prior to the surgery, informed consent was obtained from all the patients, with donor identities kept confidential. Within 24 hrs after the surgery, the skin was placed in plastic bags and frozen at -20°C until used. The research ethics committee of the North-West University (Potchefstroom Campus) authorized ethical approval for the procurement and use of the donated skin under reference number NWU-00114-11-A5 (2011-08-25).
Prior to preparation, the skin was thawed at room temperature. Thereafter the skin was removed from the plastic bags and cleaned using distilled water and paper towels to remove any blood and other remaining fat tissue left on the skin surface. Using an electric dermatome model 8821 (Zimmer, Inc., Warsaw, Ind. USA), a layer of skin with a thickness of 400µm and a width of 2.5cm was removed. The excised skin was placed dermal side down on filter paper and stored in aluminum foil at -20°C until used. One hour prior to the permeation studies, the skin was thawed at room temperature and cut into circular pieces before being placed on the diffusion apparatus.

2.7.2 Skin diffusion studies

Vertical Franz diffusion cells with an active diffusion area of 1.13 cm² and receptor volume of 2 ml were used for the in vitro permeation studies. To maintain constant stirring of the PBS (pH 7.4) receptor solution, a small magnetic stirrer bar was placed in the receptor compartment of each Franz cell. Thereafter, the dermatomed skin was placed between the receptor and donor compartments with the stratum corneum facing the donor compartment. The two compartments were subsequently sealed with Dow Corning® high vacuum grease and clamped together using a horseshoe clamp to prevent leakage.

The donor compartments were filled with 1ml of the donor solution, preheated to 32 °C in a water bath to match normal human skin temperature. Parafilm® was used to cover the donor compartments to prevent evaporation of the donor solutions during the diffusion. The receptor compartments were filled with 2 ml PBS (pH 7.4), preheated to 37 °C, taking care that no air bubbles formed underneath the skin. The receptor compartments were submerged in water within a water bath set at 37°C to achieve a skin surface temperature of 32°C. After 12 hrs, the entire volume of the receptor compartment was withdrawn for HPLC analyzed.

Two diffusion studies were performed using the method described above. For the first study, the permeation of the sinigrin from sinigrin-phytosome complex was determined (n = 6). For
the second study, i.e. the control study, the donor phase consisted of sinigrin dissolved in distilled water (n = 7).