Microemulsion assisted transdermal delivery of a hydrophilic anti-osteoporotic drug: Formulation, in vivo pharmacokinetic studies, in vitro cell osteogenic activity

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ABSTRACT
Gastrointestinal adverse effects such as esophageal irritation and ulcers are the major disadvantages of the oral administration of alendronate (ALD) and nitrogen-containing bisphosphonate. We hypothesized that the transdermal delivery of ALD via water in oil (w/o) microemulsion might help to prevent the aforementioned side effects without compromising the efficacy. The pseudo-ternary phase diagrams were constructed with varying ratios of surfactant mixture and oil to recognize the concentration range of excipients required to form a monophasic microemulsion. Globule size, morphology transmission electron microscopy, and thermal behavior differential scanning colorimetry of drug-loaded microemulsion were investigated. The in vitro permeation studies revealed significantly enhanced permeation of ALD through microemulsion than pure solution across the rat skin (p < 0.01). In an in vivo pharmacokinetic study in Wistar rats, microemulsion achieved around two folds higher bioavailability than pure drug solution (p < 0.05) when given in equal doses (30 mg/kg). Cell viability assay with human osteoblastic osteosarcoma (MG-63) cells exhibited the positive effects of ALD microemulsion on cell growth. Moreover, alkaline phosphatase and mineralization studies proved that microemulsion as a carrier retains distinct osteogenic properties of ALD. Overall, these outcomes demonstrated that the w/o microemulsion as a transdermal carrier is a promising approach for the effective delivery of ALD, bypassing the adverse effects associated with oral administration.

INTRODUCTION
Bisphosphonates (BPs) are pyrophosphate analogs that are prescribed as first-line drugs for the prevention and treatment of bone disorders such as Paget’s disease, hypercalcemia of malignancy, osteopenia, and osteoporosis (Drake et al., 2008; Ezra and Golomb, 2000). BPs act by inhibiting the mevalonate pathway in bone resorption, which is a crucial step in the bone remodeling process (Lambrinoudaki et al., 2006; Schenk et al., 1986; Van Beek et al., 2003). The majority of orally administered BPs such as alendronate (ALD) has serious gastrointestinal adverse conditions such as irritation of the mucosal wall of GIT, gastric ulcers, and esophagitis. Moreover, ALD has very low (not more than 2%) bioavailability, and its systemic absorption is further hampered by the presence of food. To avoid all aforementioned side effects, the dose of ALD is advised to be taken empty stomach in the morning in an upright sitting position with a big glass of water. This practice helps in reducing the irritation of mucosal lining but can be hard to comply with by elderly patients (Graham, 2002; Naniwa et al., 2008; Porras et al., 1999). This crisis raises the need for the development of a carrier system and/or a choice of a different route that can efficiently improve the bioavailability of ALD, escaping its oral side effects and keeping its osteogenic activity intact.

Drug molecules currently administered via the transdermal route fall within a narrow range of molecular weight, typically less than 500 g/mol (Benson, 2005; Marwah et al., 2014). Thus, ALD (molecular weight 325 g/mol) can be the potential candidate for transepidermal delivery. Transdermal application has numerous potential benefits over oral delivery such as evading GIT side effects, comfort in an application, and ultimately better...
patient compliance (Katsuni et al., 2012), especially the elderly patients in this case. The key step in the transdermal application of ALD, a hydrophilic candidate, is delivering it through stratum corneum in an adequately high amount to exert therapeutic effect (Choi et al., 2008; Yano et al., 1986). Many research studies have explored and established microemulsions as an effective carrier to deliver polar drugs across the skin for local and systemic effects (Alkrad et al., 2019; Kaur et al., 2018; Kumbhar et al., 2020; Talhouni et al., 2019). The mounting attention given to microemulsion, as a delivery system, stems from some of their peculiar characteristics such as the larger surface area of the inner dispersed phase which gives better drug release, improved penetration capacity across biological membranes, and high stability (Hashem et al., 2011; Nastiti et al., 2017; Sapra et al., 2014). Besides, the strategic selection of components of the microemulsion can further enhance the penetration of drugs across the skin without causing any skin irritation. In this study, isopropyl myristate (IPM) is selected as an oil phase as its usage in the transdermal system has add-on benefit being biocompatible permeation enhancer (Furuishi et al., 2019; Kantaria et al., 2003; Zidan et al., 2017). Aerosol OT (bis-2-(ethylhexyl) sulfosuccinate, AOT) has been comprehensively used with IPM to formulate transdermal microemulsions due to its non-irritant properties (Anjum et al., 2016; Changez and Varshney, 2000; Dib et al., 2020; Liebig et al., 2018). AOT facilitates solubilization of a sizable extent of water with oil even when the aqueous phase has higher drug loading. Moreover, the synergistic influence on water solubilization ability was observed when AOT was used in combination with a nonionic surfactant like labrasol/tweens (Kantarai et al., 2003; Yanyu et al., 2012).

Taking into account the abovementioned specifics, we conjectured that the encapsulation of ALD in IPM/AOT/ labrasol w/o microemulsion may result in enhanced permeation of ALD across the epidermal barrier and can achieve greater bioavailability than oral administration of the same. Furthermore, we hypothesized that ALD microemulsion can demonstrate osteogenic activity on osteoblastic cells.

MATERIALS AND METHODS

Materials

ALD was received as a gift sample from Aurobindo Pharmaceuticals, Hyderabad, India. IPM and dioctyl sulfosuccinate (AOT) were purchased from Sigma Aldrich, Mumbai, India. Labrasol was generously donated by Gattefosse, India. All the solvents used were of high performance liquid chromatography (HPLC) grade, and chemicals were of analytical grade. Freshly collected Milli-Q water (Millipore, Billerica, MA) was used in the preparation of aqueous mobile phase of HPLC analysis.

Construction of pseudo-ternary phase diagrams

To record the concentration range of each microemulsion component, pseudo-ternary phase diagrams were constructed with the titration method. Five phase diagrams, with IPM as oil, were studied with 1:1, 2:1, 3:1, 4:1, and 5:1 weight ratios of AOT (surfactant) to labrasol (co-surfactant). The ratio of surfactant mixture (AOT + labrasol) to oil was varied at 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1 for each phase diagram. An aqueous solution of the drug was added in a dropwise manner to the surfactants and oil mixture under moderate magnetic stirring, and a solution turning slight turbid is considered as an endpoint (Yanyu et al., 2012).

Preparation of microemulsion

The compositions of microemulsion with varying concentrations of components were selected from the phase diagram. The microemulsion was prepared by gradually titrating a weighed amount of oily mixture of IPM, AOT, and labrasol with an aqueous solution of ALD (Sintov and Botner, 2006). The microemulsion was formed spontaneously at room temperature as a clear monophase liquid.

Physical appearance

The prepared microemulsion (blank and drug-loaded) was scrutinized through visual inspection of their homogeneity, phase separation (if any), and consistency.

Globule size

The microemulsion was characterized for average globule size with Malvern Zetasizer 7.12 (Malvern Instruments Ltd., Malvern, UK).

Transmission electron microscopy (TEM)

The morphology of microemulsion was observed by TEM (TEM JEM-2010-Japan Electron Optics Laboratory Company, Tokyo, Japan). A single drop of optimized formulation was put on a copper grid and then dried at room temperature. Pictures were recorded by TEM with suitable magnification.

Drug content

The drug content in microemulsion was determined by the following previously reported multistep derivatization process for ALD and analyzing a sample by high-performance liquid chromatography (Meng et al., 2010).

Refractive index

The refractive indices for both blank and ALD microemulsion were determined using a refractometer (Fisher Scientific, Japan) in triplicates.

Viscosity

The viscosities of blank and drug-loaded microemulsions were measured with a Brookfield cone and plate rheometer (Brookfield Engineering, Middleborough, MA) with a No. 1 rotor set at 60 rpm. The temperature was controlled at 25°C.

Density

The density of blank and ALD added microemulsion was measured using a pycnometer. Precisely measured 10 ml of sample microemulsion was taken in a container and accurately weighed, and the density was calculated. The experiment was carried out in triplicates for each sample.

Electrical conductivity

The electrical conductivity of the microemulsion samples was measured by Riai CM/100 conductivity meter (Yellow Springs Instruments, Yellow Springs, OH). The tests were performed in triplicates at room temperature.
DSC

DSC analysis was performed (STAR®e, Mettler Toledo GmbH Analytical, Giessen, Germany) to examine the thermal behavior of water in the microemulsion. The samples of blank and ALD loaded microemulsions were accurately weighed (5–10 mg) into 40-0 µl aluminum pans, and the pans were sealed, kept under sub-zero temperature (~70°C) overnight, and scanned between ~-40°C and 150°C, at a heating rate of 1°C/minute. A blank sealed aluminum pan was used as a reference.

Thermodynamic stability studies

**Heating cooling cycle**

Blank and ALD microemulsions went through 6 cycles, where they were kept at 40 ± 2°C and then at 4 ± 2°C for at least 48 hours (Hamed et al., 2019).

**Centrifugation test**

Microemulsions underwent centrifugation at 3,500 rpm for 30 minutes and were scrutinized for any drug precipitation or phase separation or any color/consistency changes (Hamed et al., 2019).

**Freeze-thaw cycles**

Formulations were stored between -25 ± 2°C and 25 ± 2°C at least for 48 hours and were examined for any drug precipitation or phase separation or any color/consistency changes (Hamed et al., 2019).

**Stability studies**

The physical and chemical stability of ALD-loaded microemulsion was studied by keeping samples at 25 ± 2°C, relative humidity 60 ± 5% for 3 months. The samples were withdrawn at selected time intervals and were tested visually for globule size. Drug content was assessed by HPLC (Pakpayat et al., 2009).

**In vitro permeation**

**In vitro** permeation profiles were evaluated through excised rat skin, mounted on Franz diffusion cells (area 0.95 cm²) with stratum corneum area facing toward donor chamber. Accurately 3-4 ml of phosphate buffer solution (phosphate buffer saline (PBS) pH 7.4) was filled in the receiver section of diffusion cells maintained at 32 ± 0.5°C and was stirred magnetically at 300 rpm. Precisely measured samples of microemulsion (dose: 0.1, 0.5, or 1 mg of ALD) were placed in the donor compartment above the stratum corneum side of rat skin. The permeation profiles of microemulsions were compared with an aqueous solution of ALD (dose: 1 mg) placed in a separate diffusion cell. The aliquots of acceptor media (500 µl) were collected at regular intervals, and each aliquot was substituted with an equal volume of fresh pre-heated PBS. An ALD concentration was determined using the reverse phase HPLC method, and the quantity of ALD permeated was determined (Meng et al., 2010).

**In vivo pharmacokinetic studies**

Animals were divided into five groups (n = 6). Hairs were removed from the skin (about 10 cm²) of three groups of rats. These groups received ALD microemulsion transdermally at a dose of 30, 25, or 20 mg/kg. The fourth group of rats received an ALD solution orally at a dose of 30 mg/kg. Alendronate solution (1 mg/kg) was administered intravenously to rats in the fifth group. At pre-determined time intervals, the blood samples were collected, and plasma was isolated instantly by centrifugation at 3,000 rpm for 15 minutes. Alendronate plasma concentrations were measured by HPLC (Meng et al., 2010). Pharmacokinetic parameters were calculated for each individual set of data by noncompartmental analysis using WinNonLin version 4 (Pharsight, Mountain View, CA). The absolute bioavailability was evaluated for transdermal as well as oral delivery of ALD.

**MTT assay**

The influence of ALD (pure drug and microemulsion) on cellular viability was studied with MG-63 osteoblast-like cells (NCCS, Pune, India) by culturing them as defined earlier (Kim et al., 2012). Cells were collected after reaching confluence and plated in 96-well plates. Cells were incubated at 37°C in the incubator with 5% CO₂ and were washed with PBS. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed by placing cells into each well of 96-well plate, incubating them overnight, adding 0.5 mg/ml of MTT into each well, and further incubating them for 4 hours at 37 ± 0.5°C. The culture medium was then aspirated, and the produced formazan crystals were dissolved by adding 150 µl of dimethyl sulfoxide (DMSO) (Ha et al., 2003). The supernatant was collected, and the colorimetric changes were measured at 490 nm in a microplate reader. MTT assay was carried out with four groups: control, ALD solution, blank microemulsion, and ALD microemulsion. The results are represented as the percentage optical density of samples considering the control group as 100%.

**Alkaline phosphatase (ALP) activity**

ALP activity was analyzed in four different groups: control, ALD solution, blank microemulsion, and ALD microemulsion. The MG-63 cells were plated in well plates at a density of 1 × 10⁵ cells. After overnight incubation, ALD microemulsion/ALD pure solution/blank microemulsion was added, and the cultures were continued for 3, 7 and, 10 days. Cells were lysed after completion of the study, cell lysates were then centrifuged for 3 minutes (15,000 rpm, 4 ± 0.5°C), and the supernatants were separated. 1N NaOH was added after 30 minutes of incubation of supernatant with p-nitrophenyl phosphate (30 minutes, 37 ± 0.5°C). ALP activity was determined by measuring the conversion of p-nitrophenyl phosphate to p-nitrophenol. An optical density was determined at a wavelength of 405 nm.

**Mineralization**

Mineralization study was carried out in four different groups: control, ALD solution, blank microemulsion, and ALD microemulsion. The MG-63 cells were plated in 24-well tissue culture plates (density 1 × 10⁴ cells). After overnight incubation, blank microemulsion/ALD microemulsion/ALD pure solution was added, and the plates were kept for incubation for either 14 or 21 days separately at 37 ± 0.5°C. On completion, 0.5N HCl was added to the cells, cell lysates were centrifuged (3 minutes, 15,000 rpm, 4 °C), and the supernatant was used for the measurement of calcium deposition using QuantiChromTM Calcium Assay
Kit (DICA-500, BioAssay Systems, Hayward, CA). The protocol provided by the manufacturer was followed. An optical density was determined at a wavelength of 612 nm.

RESULTS AND DISCUSSION

Pseudo-ternary phase diagrams and preparation of microemulsion

It is easier to get an idea of a concentration range of different components required for a monophasic microemulsion through ternary phase studies. Figure 1 shows the results of phase studies performed with IPM, AOT, and labrasol. Oil, water, and surfactant mixture were denoted as oil, water, and S_mix in the phase diagram, respectively. Each diagram describes the isotropic microemulsion region for individual ternary mixture denoted by the shaded region. The sizeable monophasic region was obtained in each plot, and it was majorly confined to the oil S_mix axis, which indicates formation w/o microemulsion. An ample amount of water was solubilized with the help of the chosen surfactant mixture without resulting in phase separation. Mitra and Paul have previously reported an improvement in water solubilization ability in w/o microemulsion with a mixture of surfactants comprising of AOT and non-ionic surfactants (Mitra and Paul, 2005). The addition of non-ionic surfactant labrasol to AOT played a major role in increasing solubilization of a high amount of water and hence resulted in a wide microemulsion region. We observed that with an increase in the ratio of AOT:labrasol up to a certain extent (from 1:1 to 4:1), the monophasic microemulsion region grows in the area, beyond which microemulsion domain (at 5:1) reduced a little. This behavior is explained by few studies which clarify that the expansion of the microemulsion region depends on the certain mole fraction of a non-ionic surfactant and can increase or decrease beyond it (Kantaria et al., 2003; Mitra and Paul, 2005). On the other hand, Kantaria found no clear indication between this particular pattern with hydrophilic-lipophilic balance (HLB) of surfactants and recommended to perform ternary studies with multiple ratios of surfactant mixture, similar to we did here, to get an exact idea, which surfactant ratio would be the best to use for the formation of w/o microemulsion.

The inspection of phase diagrams concludes that, at AOT/labrasol ratio of 4:1, the maximum water solubilization was possible. A higher percentage of water in stable microemulsion can prove beneficial in incorporating water-soluble drugs such as ALD. The final formulation was prepared by keeping the surfactant weight ratio at 4:1. The composition of ALD microemulsion is shown in Table 1. The final drug concentration in microemulsion was around 0.2%w/w.

Table 1. Percentage composition of blank microemulsion.

| Excipients          | % of component (w/w) |
|---------------------|-----------------------|
| Oil (IPM)           | 44                    |
| Surfactant (AOT)    | 28                    |
| Co-surfactant (labrasol) | 7                  |
| Water               | 21                    |

aIPM = isopropyl myristate; AOT = Dioctyl sodium sulfosuccinate (Aerosol OT).

Figure 1. Ternary phase diagrams for water–AOT/labrasol–IPM combination with varying ratios of AOT:labrasol (a) 1:1, (b) 2:1, (c) 3:1, (d) 4:1, (e) 5:1.
Physical appearance

Microemulsions appeared as clear and transparent homogenous liquid with no turbidity or phase separation. There was no visible drug precipitation in ALD microemulsion. These results impart that the prepared microemulsion was a stable monophasic liquid.

Globule size

Globule size analysis exhibited a mean globule size of microemulsion in nanosize range with a mean diameter of 93.21 ± 5.95 nm. The polydispersity index was observed to be on a lower side (<0.3) which indicates uniform globule sizes within the formulation.

Transmission electron microscopy

TEM result for the ALD microemulsion is shown in Figure 2. It displayed spherical-shaped droplets of microemulsion with the size range around 100 nm similar to indicated by globule size studies.

Drug content

The drug content of the microemulsion was found to be 89.56 ± 4.2%w/w.

Refractive Index (RI)

The refractive index of drug-loaded microemulsion stayed unchanged as that of blank microemulsion (Table 2).

| Parameter         | Blank microemulsion | ALD microemulsion |
|-------------------|---------------------|-------------------|
| Refractive index  | 1.431 ± 0.011       | 1.434 ± 0.019     |
| Viscosity (mPas)  | 7.67 ± 0.51         | 7.72 ± 0.48       |
| Density (gm/ml)   | 0.939 ± 0.022       | 0.941 ± 0.036     |
| Conductivity (μS/cm) | 500.8 ± 53.9   | 523.5 ± 27.9      |

Values are given as mean ± standard deviation, n = 3.

Viscosity

The blank and ALD-loaded microemulsion displayed low viscosity (Table 2) which is the characteristic of microemulsion (Junyaprasert, 2008).

Density

The density of blank and ALD microemulsion remains unchanged as a small amount of the drug did not have any significant effect (p > 0.05) on the density of microemulsion (Table 2).

Electrical conductivity

Low electrical conductivity is a typical property of w/o microemulsion due to the presence of a non-aqueous outer phase. As electrical conductive component in a microemulsion is water, it is present as the inner phase, and the low electrical conductivity is observed when w/o microemulsions are concerned (Cao et al., 2019; Kantaria et al., 2003). The conductivity of both, blank and drug-containing w/o microemulsion, was found to be quite low (Table 2).

Differential scanning colorimetry

The samples of pure IPM, blank, and ALD-loaded microemulsion were compared for a cooling–heating cycle with the help of DSC. Some important characteristics of thermogram related to the melting or freezing of oil/water points were observed. In o/w microemulsion, water is present as the continuous phase, weakly bound to surfactant, termed as free water, and freezes at the temperatures close to 0°C, whereas the water existing as the dispersed phase in w/o microemulsion is strongly bound to the surfactant at the interface and is termed as bound water. Such water freezes at the temperature below 0°C. Hence, studying the thermal pattern of bound water as the dispersed system is a simple, easy, and supplemental tool to learn about the microstructures of microemulsions (Liu et al., 2009). In Figure 3, endothermic peak appearing above zero temperature (5.4°C) represented the melting of the oil, and this peak is prominent in all three DSC curves. A very weak endothermic peak below zero temperature (~5.5°C) was attributed to the melting of bound water in the dispersed phase. This weak endothermic peak was absent from the thermogram of pure IPM. The DSC curves of blank microemulsion and their drug-loaded counterpart were generally similar; however, slight shifts in the thermal events were observed. This may be because the drug has a certain effect on a surfactant film or the interaction between surfactant and water molecules (Boonme, 2007; Podlogar, 2004). The results of DSC studies are in agreement with observations from conductivity and RI and confirm the type of prepared microemulsion as w/o.
Thermodynamic stability studies

The prepared microemulsion could survive a thermodynamic stability test without any drug precipitation and phase separation. Low interfacial tension due to surfactant blend and smaller globule size could be vital reasons for the stability of microemulsion against extreme conditions (Russell-Jones and Himes, 2011; Yanyu et al., 2012).

Stability study

As stability study is a crucial parameter for the evaluation of microemulsion, it was performed effectively for 3 months, and the satisfactory results were obtained with globule size and drug content (Table 3). No drug precipitation or visual aggregation was reported as well. Hence, it is suggested that the prepared w/o microemulsion is stable at least for 3 months.

In vitro permeation

Alendronate microemulsion in varying doses of 0.1, 0.5, and 1 mg was applied to rat skin, and the permeation behavior of these test samples was compared with an aqueous solution (dose: 1 mg) of ALD. The lowest amount of ALD, i.e., only 17 ± 5.45 μg/cm² was permeated through rat skin with ALD microemulsion having a dose of 0.1 mg (Fig. 4a), in 24 hours. Similarly, with a medium dose of ALD microemulsion (dose 0.5 mg), a significantly lower amount of drug was permeated (121± 9.01 μg/cm²) when compared to microemulsion with a 1 mg dose of ALD (350 ± 42.69 μg/cm², p < 0.05), in 24 hours. Earlier research work, performed by Russell Jones and Himes, has stated that concentration gradient acts as a key factor in the absorption of hydrophilic drugs across the skin, and passive diffusion is the principal mechanism in the process (Russell-Jones and Himes, 2011). Hence, a higher drug permeation through w/o microemulsion was observed with a larger dose of ALD. In the case of ALD solution (dose 1 mg), a significantly low amount of ALD got permeated at the end of 24 hours (102 ± 35.4 μg/cm², p < 0.05) when compared to ALD microemulsion (dose 1 mg). Alendronate microemulsion with medium dose (0.5 mg) achieved a little higher permeation than ALD solution but lacked significance (p > 0.05). These better permeation profiles of ALD through w/o microemulsion can be attributed to the different components of microemulsion which can act as permeation enhancers. The inclusion of IPM in the microemulsion can considerably boost the permeation of drugs across the skin than many other commonly used pharmaceutically accepted oils (Zhang et al., 2011) by modulating the lipid barrier of the skin (Suh and Jun, 1996). Besides, several studies advocate the use of AOT in w/o microemulsion to augment the permeation of drugs through the rat skin (Gupta et al., 2005). The lipophilic portion of the AOT works together with keratinous proteins of the skin causing the softening of the stratum corneum which eases the permeation process (Changez and Varshney, 2000). Non-ionic surfactants too, labrasol in this study, contribute to permeation enhancement, by increasing the fluidity of stratum corneum (Nokhodchi et al., 2003). Moreover, the nanosize of the microemulsion droplets increases the chances of their adherence

| Time (months) | Globule size (nm) | Drug content (%) |
|---------------|-------------------|------------------|
| 0 Months      | 93.21 ± 5.9       | 89.56 ± 4.2      |
| 1 Months      | 94.29 ± 3.6       | 89.76 ± 5.6      |
| 2 Months      | 93.93 ± 4.5       | 88.81 ± 3.5      |
| 3 Months      | 95.96 ± 3.8       | 88.65 ± 6.4      |

Values are given as mean± standard deviation, n = 3.
to the skin which eventually can result in better permeation (Yanyu et al., 2012; Kogan and Garti, 2006). This confirms that the submicron globule size of microemulsion along with the use of optimal excipients, which acted as permeation enhancers, improved the permeation of ALD along rat skin than a solution of ALD. We hypothesized that, noteworthy, improvement in skin permeation with w/o microemulsion would favorably increase the bioavailability of ALD when applied transdermally.

**In vivo pharmacokinetic study**

A pharmacokinetic study was performed to support *in vitro* data and to investigate if dose reduction of ALD could be possible with improvement in bioavailability on transdermal delivery of w/o microemulsion. Plasma concentration–time profiles and post-administration of ALD in rats via various administration routes are shown in Figure 4b, and the standard pharmacokinetic parameters are shown in Table 4. The pharmacokinetic profiles of transdermal ALD microemulsion at three different doses (at a dose of 20, 25, and 30 mg/kg) were compared with intravenous (dose 1 mg/kg) and oral (dose 30 mg/kg) administration of ALD. On the transdermal application of microemulsion, the plasma concentrations of ALD gradually increased for the first 3 hours and were at an almost constant level up to 12 hours. This might be due to the continuous and controlled delivery of ALD from the w/o microemulsion (Zhao et al., 2006). This observation was unchanged for all transdermal microemulsion groups with different doses of ALD. An intravenous administration of ALD demonstrated a quick and steep rise in plasma concentration and subsequent disappearance from the bloodstream. The oral administration of ALD solution (30 mg/kg) followed a similar pattern as that of intravenous administration with quickly attending C_{max} (285 ± 89.41 ng/ml at T_{max} 1 hour) than transdermal microemulsion (T_{max} 3 hours) and fading afterward from the bloodstream. This difference in the T_{max} could be due to the delay in the permeation.
of ALD due to multiple layers of the skin (Gannu et al., 2010). It was noted that the AUC_{0-24} of ALD increased with ascending order of the dose of ALD microemulsion (20 < 25 < 30 mg/kg). Dosing ALD microemulsion (30 mg/kg) via transdermal route led to around 2-fold higher AUC_{0-24} than ALD oral solution (30 mg/kg, p < 0.05). Furthermore, ALD transdermal microemulsion at dose of 25 mg/kg resulted in higher AUC_{0-24} (even with a lower dose than that of the ALD oral solution (30 mg/kg) but failed to report any significance (p > 0.05). A higher bioavailability was achieved with ALD w/o microemulsion (dose 20/g/kg; 2.3%, and 30 mg/kg; 3.58%) when compared with ALD oral solution (dose 30 mg/kg; 1.85%).

Based on the results of the pharmacokinetic study, the w/o microemulsion proved to be an effective carrier system to deliver ALD across the skin. Oil from a continuous phase of w/o microemulsion is compatible with sebum from hair follicles. It is reported that, for hydrophilic drugs such as ALD, hair follicular route is the main path for permeation through the skin (Teichmann et al., 2006). The globule size of the microemulsion carrier is generally smaller than the size of the opening of a hair follicle and, hence, can help the penetration of drugs. The hair follicle provides a larger surface area with compromised stratum corneum (Abd et al., 2018; Blume-Peytavi and Vogt, 2011), and a thick network of blood capillaries around the follicle enhances the systemic absorption of the drug (Lademann et al., 2009). Furthermore, using permeation enhancers in the microemulsion, in the form of oil and surfactants, can contribute to the increased permeation of drugs on transdermal application (Gannu et al., 2010; Setya et al., 2017). These individual microemulsion components, when taken together as a chemical mixture, can act synergistically for the enhancement

| Parameters     | Dose (mg/kg) | C_{max} (ng/ml) | T_{max} (hr) | AUC_{0-24} (ng.h/ml) | BA (%) |
|----------------|--------------|-----------------|--------------|----------------------|--------|
| ALD IV 1 mg    | 1            | 285 ± 89.41     | 1            | 1,671.5 ± 306.81     | 1.85   |
| ALD oral 30 mg | 30           | 72 ± 15.63      | 3            | 931.12 ± 271.15      | 1.48   |
| ALDME TD 20 mg | 20           | 108 ± 10.39     | 3            | 746.25 ± 111.83      | 2.3    |
| ALDME TD 25 mg | 25           | 132 ± 21.62     | 3            | 1,154.5 ± 184.23     | 3.58   |
| ALDME TD 30 mg | 30           | 1,798.5 ± 203.98| 3            | 1,798.5 ± 203.98     | 3.58   |

ALD IV = alendronate solution intravenous; ALD oral = alendronate solution oral; ALDME TD = alendronate microemulsion transdermal.

Values are given as mean ± standard deviation, n = 3.

Figure 5. Proliferation of osteoblast-like cells (MG-63 cells) treated with blank microemulsion (ME), pure drug alendronate (ALD), and alendronate microemulsion (ALD ME) for 48 and 72 hours, expressed as a percentage of optical density relative to control (medium only) of 100%, using MTT assay (mean ± S.D, n = 3, *p < 0.05 compared with the control group).
of transdermal drug delivery (Karande and Mitragotri, 2009). Based on pharmacokinetic profiles of ALD given by different routes, we observed that reduction in the dose of ALD transdermal microemulsion (dose 25 mg/kg) gave an insignificantly different plasma profile than ALD oral solution (dose 30 mg/kg, \( p > 0.05 \)). On the other hand, ALD transdermal microemulsion with the same dose (30 mg/kg) as that of the oral solution (dose 30 mg/kg, \( p > 0.05 \)) resulted in a significant increase in exposure (AUC\((0-24)\) and the bioavailability (\( p < 0.01 \)). Comparing the results of a pharmacokinetic study with that of skin permeation studies, a similar trend is observed and confirmed the selection of particular microemulsion excipients assisted in improving the permeation of ALD with microemulsion. This research work states that the use of a lipid carrier for improving the transepidermal permeation can be a sound method for the proficient delivery of hydrophilic drugs such as ALD across the skin and can probably be used to avoid toxicity related to its oral administration.

### MTT assay

Cultured cell lines are generally utilized to scrutinize the vital aspects of drug metabolism linked to toxicity. We used MG-63 cells in this study as they are immature osteoblastic cells, which enables studying the effect of ALD on the initial developmental stages of cells (Xiong et al., 2009). MTT assay as a cell viability study was conducted to estimate the effect of ALD and ALD microemulsion on MG-63 osteoblast-like cells (Fig. 5). We represented the data relative to the optical density of the control group as 100%. Treatment with ALD was successful in promoting cell proliferation, indicating that the test samples were not cytotoxic. In ALD-treated groups (ALD solution and microemulsion), cell number was greater at 48 hours and significantly higher at 72 hours of treatment (\( p < 0.05 \)) than in the untreated groups (control and blank microemulsion). The increase in cell number was the greatest for ALD microemulsion after 72 hours (13% increase above control cells). When

![Figure 6](image-url)

**Figure 6.** (a) ALP activity of osteoblast-like cells (MG-63 cells) treated with medium only (control) blank microemulsion (ME), pure drug alendronate (ALD), and alendronate microemulsion (ALDME) after 3, 7, and 10 days of incubation (mean ± S.D, \( n = 3 \), \( * p < 0.05 \) compared with control and ME group, \( a p < 0.01 \) compared with control and ME group). (b) Calcium deposition of osteoblast-like cells (MG-63 cells) treated with medium only (control) blank microemulsion (ME), pure drug alendronate (ALD), and alendronate microemulsion (ALDME) after 14 and 21 days of incubation (mean ± S.D, \( n = 3 \), \( * p < 0.05 \) compared with control and ME group, \( a p < 0.01 \) compared with control and ME group).
comparing with ALD solution, the viability of cells cultured with ALD microemulsion was higher but lacked significance ($p > 0.05$). Similar effects of ALD formulations against osteoblastic cells are reported in earlier works highlighting its osteogenic ability (Ezzati et al., 2014; Im et al., 2004; Kim et al., 2012). Im et al. described the proliferative and maturation inducing effects of ALD and risedronate on MG-63 osteoblast-like cells (Im et al., 2004). Ezzati et al. reported that the nanoformulation of ALD was not only cytocompatible but also increased viability of cultured cells than ALD pure drug (Ezzati et al., 2014). Kim et al. exhibited similar activity of ALD with no cytotoxicity (Kim et al., 2012). Although the primary mechanism involved in bisphosphonate-induced cell proliferation is unknown, few researchers considered that extracellular signal-regulated kinases can play a key role in the same (Mathov et al., 2001). Few studies observed inhibitory or no effects of bisphosphonates on the viability of human osteoblastic cells (Garcia-Moreno et al., 1998; Reinholz et al., 2000). The explanations for these contradictory observations might be related to the bisphosphonate analog and its concentration range used in the study. The overall time of treatment and type of cells could also be responsible for the differing effects.

From the results of the MTT assay, we speculated that, along with proliferation, ALD microemulsion can induce maturation and osteogenic activity in MG 63 osteoblastic cells.

**ALP activity and mineralization**

ALP is a definite index for the estimation of osteogenic activity and tissue differentiation. We evaluated the influence of ALD on the initial differentiation of osteoblasts through ALP activity at 3, 7, and 10 days (Fig. 6a). We noted an increase in the ALP activities of MG-63 cells of all substrates for 10 days of the incubation period. At 3 days of incubation, the ALP levels of cells cultured without the drug (i.e., control or blank microemulsion) did not differ significantly from cells cultured with ALD microemulsion/solution (Fig. 6a, $p > 0.05$). On the contrary, MG-63 cells cultivated with ALD microemulsion or solution showed a significant increase in ALP activity at day 7 ($p < 0.05$) and day 10 ($p < 0.01$) of incubation than that of untreated cells. The maximum levels of ALP were noted with the cells cultured with ALD microemulsion at 10 days of incubation (Fig. 6a). In an earlier study by Kim et al., the ALP levels of osteoblast-like cells treated with ALD were found to be significantly higher in comparison to the control group (Kim et al., 2012). Xiong et al. positively correlated the osteogenic activity of ALD with ALP activities of osteoblastic cells. They have shown that ALD upregulated ALP levels in cells along with the increased expressions of genes of bone turnover markers such as osteocalcin, bone morphogenetic protein and collagen (Xiong et al., 2009). Wang et al. observed that ALD is capable of increasing ALP activity in osteoblastic cells other than MG-63 (Wang et al., 2010). Here, we have shown that ALD enhances differentiation and bone formation by increasing ALP activity in osteoblastic cells, which is also supported by the previous research works (Kim et al., 2010; Moon et al., 2011; Shi et al., 2009).

A late differentiation of osteoblasts was studied by determining the calcium deposition by MG-63 cells on culturing with ALD solution and microemulsion for 14 and 21 days (Fig. 6b). Alendronate significantly raised calcium deposition in both substrates (ALD solution and microemulsion) after treatment for 14 and 21 days when compared with cells cultured without the drug (control and blank microemulsion, $p < 0.05$). Results confirmed that ALD microemulsion encourages the maximum deposition of a mineral matrix in cultured osteoblast-like cells (Fig. 6b). BPs are known to promote the mineralization of cell culture through various cellular pathways including inhibition of mevalonate, protein tyrosine phosphatase pathway, or acceleration of non-RAS-MEK-ERK½ cbfa 1 pathway (Fujita et al., 2001; Itoh et al., 2003; Reinholz et al., 2002). These results are in agreement with those of Xiong et al. study which indicates that ALD augments the development of MG-63 cells to the mineralization stage (Xiong et al., 2009).

Results from mineralization study support the outcomes of MTT and ALP studies. Taken together, these findings recommend that ALD microemulsion can promote cellular functions of osteoblast-like cells and can prompt their differentiation and maturation. The results in this study support the theory that ALD has anabolic and osteogenic effects on osteoblasts.

**CONCLUSION**

The present study successfully confirmed that the transdermal w/o microemulsion system can present a real interest in the systemic delivery of a hydrophilic drug, such as ALD. The transdermal application of microemulsion provided a better pharmacokinetic profile than oral ALD which may prove beneficial in anti-osteoporotic treatment. The results indicate that the encapsulation of ALD, a bisphosphonate, in a microemulsion can retain its osteogenic and anabolic effects. Hence, it is anticipated that the use of microemulsion as a transdermal carrier system can possibly improve the therapeutic efficacy of a bisphosphonate and can increase a patient compliance by evading severe gastrointestinal adverse effects.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

**STATEMENT OF HUMAN AND ANIMAL RIGHTS**

The protocols for animal experiments (25/1617) were approved by the Animal Experimentation Committee of the National Toxicology Centre, Pune.

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