ORIGINAL ARTICLE

Synergistic iontophoretic drug delivery of risedronate sodium in combination with electroporation and chemical penetration enhancer: *In-vitro* and *in-vivo* evaluation

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In the present investigation iontophoretic permeation of risedronate sodium (RS) through optimized gel based formulation was evaluated with respect to various electrical parameters like Current density and type of current. The study also involved enhancement the iontophoretic permeation of RS in combination with electroporation and chemical penetration enhancers with its *in-vivo* Pharmacokinetic evaluation. The permeation studies were carried through the human cadaver skin by using modified Franz diffusion cell and microcontroller based devices for iontophoresis and electroporation developed in the laboratory. *In-vivo* Pharmacokinetic studies were carried out in hairless rats. One way ANOVA followed by Tukey-Kramer test for multiple comparisons. The permeation of RS was significantly increased with iontophoresis at 0.5 mA/Cm^2 current density. The iontophoretic permeation was found depend on current density and ON:OFF ratio. The pulsatile current resulted in high permeation than continuous current. Maximum permeation was obtained at 0.5 mA/cm^2 with 1:1 pulsed current. When iontophoresis was coupled with chemical penetration enhancers and electroporation for 100 ms at 220V, synergistic enhancement in permeation was observed with shortened lag time and high flux. The required flux was achieved with area of application 1.55 cm^2. *In-vivo* studies in hairless rats revealed high $C_{max,}$ low t_{max} and increased area under the curve with electroporation, followed by iontophoresis indicated increased bioavailability. Relative bio-availability was 4.6 when calculated in comparison to passive studies.

Key words: *Electroporation, iontophoresis, penetration enhancers, risedronate sodium*

INTRODUCTION

Risedronate sodium (RS) is potent bisphosphonates derivative for clinical use in treatment of osteoporosis, Paget's disease, metastatic bone disease, hypercalcemia of malignancy and primary hyperparathyroidism. RS have very low oral bioavailability (0.63%), which may lead to highly variable dosing. Furthermore, the low oral bioavailability varies with food, calcium levels and number of other factors.[1] The oral dosing regimen of RS is also not patient friendly, the drug must be taken daily 30 min to 1 h prior to breakfast and with large amounts of water, and in addition, the patient should not lie down for 30 min after taking the drug. The drug cannot be prescribed for patients with abnormalities of the esophagus and/or the inability to stand or sit upright for at least thirty minutes; moreover drug shows transient pyrexial reaction when given by intravenously route.[2] Such issues may result in certain excluded patents and poor compliance for a chronic asymptomatic disease such as osteoporosis.

The low bioavailability is likely due to the extensive ionization and highly hydrophilic nature of bisphosphonates, which prevent the transcellular transport across intestinal epithelium and favor the paracellular route.[3]

Many attempts have been made to improve the bioavailability of bisphosphonates. Adducts of

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Access this article online

Quick Response Code:

Website: www.asiapharmaceutics.info

DOI: 10.4103/0973-8398.160312

RS and titanium dioxide particles was developed and characterized in order to improve the bioavailability after oral administration.[4] To improve the bioavailability of bisphosphonates, structural modification of the bisphosphonate molecule and use of absorption enhancers in designing of drug delivery systems have been attempted.[5] Surfactants such as sodium lauryl sulfate and medium chain fatty acid esters were found to increase bisphosphonate absorption.[6] Comparative evaluation of different enhancers was carried out by Raiman *et al*. It was observed that enhancers acting on tight junctions are most effective compared to other categories.[7]

In these attempts, however, excessive amounts of enhancers are required to attain desired absorption of bisphosphonates. The amounts of the enhancers used are too high for a dose of the medicament so that they are impossible to apply to practical oral formulations. Further, although oral formulations with a high content of enhancers are made to enhance the bioavailability of bisphosphonates, excessive amounts of the enhancer, when administered, may irritate the intestinal mucosa, incurring a problem in the safe use of the medicament.[8]

Floating (RS)-Gelucire® 39/01 matrices were formulated with aim to sustained the release of drug and lesser GI irritation.[9] Transdermal permeation of risedronate by iontophoresis was studied with reference to effect of polarity, pH, current density, drug concentration using through hairless mice skin.[10]

Iontophoresis of an other bisphosphonate derivative, etidronate has been demonstrated that the molecule can be delivered through the skin and that it remains biologically active since a large portion was located bound to bone.[11]

Iontophoresis involves the application of a small amount of physiologically-acceptable direct current (DC) to drive ionic drugs into the body.[12] US patents disclosed iontophoretic transport of bisphosphonates derivatives including RS and etidronate.[13]

Another enhancement technique which could work in conjunction with iontophoresis is the use of a high voltage electroporation pulse. Skin electroporation takes place at high transdermal voltages (>50V) and is associated with reversible changes in the skin structure.[14,15] The use of electroporation in conjunction with iontophoresis can possibly enhance transport, allow rapid delivery of a bolus dose, allow further control on modulation or programmability of delivery, and expand the scope of transdermal delivery to larger molecules such as therapeutic proteins and oligonucleotides.

In the present investigation iontophoretic permeation of RS was evaluated with respect to various electrical parameters like Current density and type of current. The study also involved enhancement the iontophoretic permeation of RS from suitable gel formulation in combination with electroporation and chemical penetration enhancers with its *in-vivo* Pharmacokinetic evaluation.

MATERIALS AND METHODS

Risedronate sodium was obtained as gift sample from Cipla Ltd., Mumbai, Carbopol-934 from Wockhardt Research Centre, Aurangabad; all other chemicals are of AR grade from Merck Chemicals, India.

Preparation of electrodes

Rod shape silver wire (1 mm diameter, 2 cm length 99.9% pure) was used as anode. Another silver wire was dipped in melted silver chloride in porcelain dish. This silver wire was connected to negative pole of the power source at one end and immersed in 0.1N HCl at other end. A gray silver chloride layer was coated on anodal silver wire and after 24 h these wires were ready for use as cathode in iontophoretic experiments.[16]

Separation of human epidermis from human cadaver skin

The human cadaver skin was obtained from Government Medical College and Hospital, Aurangabad. The epidermis was prepared as per method used by Bhatia, *et al*., which involves soaking the whole skin in water at 60°C for 45 s. The skin was removed from water, blotted dry and pin with dorsal side down. The intact epidermis was teased off from the dermis with forceps, washed with water and used in the *in-vitro* permeation studies.[17]

Development of microcontroller based electrical circuit (iontophoresis device)

The power supply used was designed and constructed using microcontroller Atmel 89S51. The input to the power supply was through a 12V DC mains adapter and the 5V DC regulated supply to the microcontroller is derived using L 7805 voltage regulator. As the application does not demand for high speed operation, 2 MHz crystal was used to provide clock pulses required for the operation of microcontroller.

The experiment was designed keeping in view the provision for applying the voltage to the cell under the following conditions.

- Continuously ON
- ON for 1 s and OFF for 1 s (pulsed current).

This feature is implemented in program in the microcontroller and for selecting one of the above programs; a thumb wheal switch is used. The thumb wheal switch is connected to the microcontroller. When the thumb wheel is set at '0' the output of the supply is continuous, when '1' is set, it is ON for 1 s and OFF for 1 s. The microcontroller output port bit P3.5 is used to control the duration, as the microcontroller cannot

source the required range of current this port pin is used to drive an electromagnetic relay. The main driven by an ICULN 2003 that works as a driver for the relay. The main function of the microcontroller is to read the value set in thumb wheal switch and according to the scheduled selected, it switches the relay making the power available at the output of relay. For indicating the ON and OFF state of the power supply a light emitting diode is provided which glows when the supply is ON and it does not glow when it is OFF. The timing of the circuit is calibrated and appropriate values are programmed in the microcontroller to provide the desired timing sequence. The supply to the relay is taken directly from the 12V supply and to the output current, a potentiometer (1 KΩ) is used. The current level desired could be selected keeping the thumb wheal switch in position and adjusting the potentiometer to give the desired value of current through the cell.

Fabrication of electrophoretic device

Electrical circuit for electroporation was fabricated on laboratory level. This circuit was made by the combination of source, regulator, transistor, 7805 series of regulator, debounce switches, zener, and microprocessor. Rectifier is to convert AC current to DC current. Zener is used to reduce the voltage. Microprocessor sends the pulse for required duration. 7805 series of regulator to converts the high volt to low volt.

The circuit was designed to follow the following parameter required to carry out experiment.

- One pulse of 110V for 10 ms
- One pulse of 220V for 10 ms
- One pulse of 110V for 100 ms
- One pulse of 200V for 100 ms.

Formulation of risedronate sodium gel

Weighed quantity of Carbopol-934 was mixed with 10 ml distilled water. In another beaker weighed quantity of RS (1% w/w) was dissolved in 5 ml distilled water. Both the solutions were mixed, and to it triethaonalamine was added with vigorous stirring. The final weight is adjusted by using distilled water.

***In-vitro* iontophoretic permeation (continuous current)**

For the iontophoretic permeation with various current densities through developed gel formulation, a Modified Franz diffusion cell was used with 20 ml of phosphate buffer (pH 7.4) as receptor medium. Human cadaver skin was mounted on the diffusion cell with the stratum corneum facing the donor compartment. The whole assembly was maintained at 37 ± 1°C on a magnetic stirrer (100 rpm) and a current was applied as per study using silver-silver chloride electrode. Samples (1 ml) were withdrawn from the receptor compartment at hourly interval for a period of 8 h and assayed for drug content by ultra violet (UV) spectrophotometer (JASCO V-630) at 262 nm. For optimization of current density, permeation studies were carried out with continuous DC of current density 0.2 mA/cm^2, 0.4 mA/cm^2 and 0.5 mA/cm^2.

Effect of pulsed current (ON:OFF ratio)

For optimization of type of current, permeation studies were carried out with pulsed DC of 0.5 mA/cm^2 current density with ON:OFF ratio of 1:1, 1:2 and 1:4 s.

Synergistic effect of various penetration enhancers to study *in-vitro* permeation of risedronate sodium gel

This study was done by using the RS gel with 5% w/w concentration of polyethylene glycol-400 (PEG-400), Ethanol, Span-20 and Tween-20 as penetration enhancers. To 5.0 g of gel containing 50 mg of RS exactly 5% w/w each of PEG-400, Ethanol, Span-20 and Tween-20 was added to it and stirred to obtain homogenous clear gel. The gel (3 gm) was placed in donor compartment and remaining study was carried out at 0.5 mA/cm^2 current density with ON:OFF ratio of 1:1.

Synergistic effect of electroporation with iontophoresis to study *in-vitro* permeation of risedronate sodium gel

In this study 3 g of gel containing 30 mg of RS with was placed in the donor compartment. The receiver solution for permeation studies was pH 7.4 phosphate buffer solution. The studies for electroporation were carried out as follows:

- 10 pulses of 110V for 10 ms at interval of 1 min
- 10 pulses of 110V for 100 ms at interval of 1 min
- 10 pulses of 220V for 10 ms at interval of 1 min
- 10 pulses of 220V of 100 ms at interval of 1 min.

Each study was followed by iontophoresis at current density 0.5 μg/cm^2 at ON:OFF ratio 1:1. The voltage was applied using silver-silver chloride electrodes. Silver wire of 4.0 cm was used as the anode and silver-silver chloride wire of 2.0 cm was used as the cathode. Samples (1 ml) were withdrawn from the receptor compartment at hourly interval for a period of 8 h. The withdrawal sample was diluted with phosphate buffer saline. buffer pH 7.4 analyses by UV spectrophotometer at 262 nm. Fresh phosphate buffer was added to replace the withdrawn sample volumes.

***In-vivo* permeation (Pharmacokinetic) studies**

To investigate the *in-vivo* potential of iontophoretic drug delivery of RS in combination with Electroporation and Chemical Penetration Enhancer the studies were carried out in hairless rats. Protocol for animal study was approved by Institutional Animal Ethical Committee (IAEC) of Y.B. Chavan College of Pharmacy, Aurangabad (Proposal no: CPCSEA/IAEC/PHARMACHEM.03/2010-11/13).

In order to restrict the area of application of the developed gel formulation and to hold the gel at the applied site, a device was fabricated with circular 'O' ring shaped spacer. A spacer was obtained by cutting the sheet of high density polyethylene of desired effective surface area for

application and capacity. The spacer was adhered to the protective impermeable backing membrane (3M Scotchpak backing-1006) using adhesive to obtain the empty device. The spacer was filled with required quantity formulated gel and active surface area was covered with fluropolymer coated films (3M Scotchpak backing-1022) as release liner using PSA (Duro Tak 387-2287, National starch and Co., USA) as adhesive on the periphery to obtain a device as shown in Figure 1.The release liner was removed before the application of the device to skin.

The anode was placed in the gel formulation whereas cathode was placed elsewhere on the body of the animal. The electrodes were first connected to the electroporation device to give 10 pulses of 220V for 100 ms at interval of 1 min, followed by iontophoresis.

Sample collected at various time intervals were analyze by using reported RP-HPLC method.[18] The mobile phase was an aqueous solution of buffer (contained 1.5 mM EDTA-2Na, 1 mM sodium etidronate, 11 mM sodium phosphate and 5 mM tetra butyl ammonium bromide as ion-pair reagent) - methanol (88:12, v/v) adjusted to pH 6.75 using 1 M NaOH. The flow rate was 1 ml min^{-1}. UV detection (λ =262 nm) was used to estimate risedronate in the concentration range of 1–5 μg ml^{-1}. Dionex-UVD 170U instrument equipped with chromeleon (Chromatography Information Management System) data acquisition and processing system software was used.

The study was carried out for 8 h in the following groups of hairless rats (n = 6).

Group-I: Passive permeation (control).

Group-II: Electroporation of 10 pulses of 220V of 100 ms at interval of 1 min followed by iontophoresis at current density 0.5 μg/cm^2 with ON:OFF ratio 1:1.

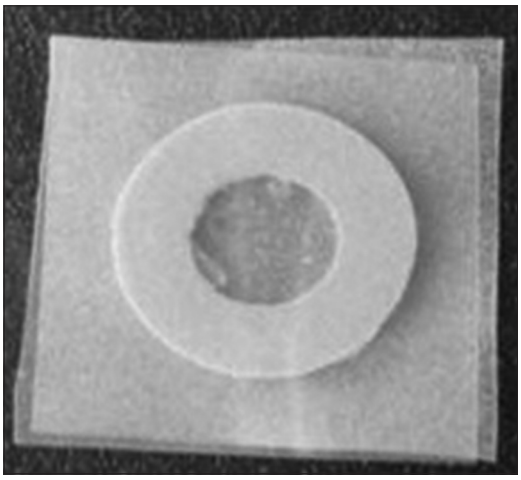

Figure 1: The spacer device to hold the gel during *in-vivo* studies (donor compartment)

The combination protocol was consisted of 10 min of electroporation following by 8 h of iontophoresis.

RESULTS AND DISCUSSIONS

Effect of current density (continuous current)

There was increase in permeation of RS with increase in current density [Figure 2]. With the current density 0.2 mA/cm^2, the flux was 124.62 μg/cm^2/h, while it was increased to 147.93 μg/cm^2/h and 194.55 μg/cm^2/h for current density 0.4 and 0.5 mA/cm^2 respectively. The flux of iontophoretic permeation study (0.5 mA/cm^2) was 194.55 μg/cm^2/h, while it was only 49.22 μg/cm^2/h, for passive study. 0.5 mA/cm^2 is reported to be the maximum tolerable current density by human being so the current density more than 0.5 mA/cm^2 was not applied.[19]

The steady state flux values obtained with different current density were compared by mean of the one way ANOVA followed by Tukey-Kramer test for multiple comparisons. There was highly significant increase in permeation between passive and iontophoretic study at 0.5 mA/Cm^2 ($P < 0.001$). As well as there was significant difference when current density was increased from 0.2 to 0.4 and 0.4 to 0.5 mA/cm^2 ($P < 0.01$). This may be attributed to the fact that increase in current density may cause increase in pore transport of the drug. This involves opening of more sweat duct resulting in more number of pores at higher current density as the pore pathway is one of the pathway assumed for iontophoresis. Since the maximum permeation was at 0.5 mA/cm^2, for further study same current density was used.

Effect of pulsed current (ON:OFF)

Use of continuous DC may result in permanent skin polarization, which can reduce the efficiency of iontophoretic delivery proportional to the length of DC application.[20] The buildup of this polarizable current can be overcome by using pulsed DC that is delivered periodically.[21] Therefore, to further increase the permeation rate and the flux of RS across the skin, pulsed iontophoresis was performed [Table 1].

The permeation of drug was studied in the pulse ratio of 1:1, 1:2 and 1:4. The permeation was found to be significantly

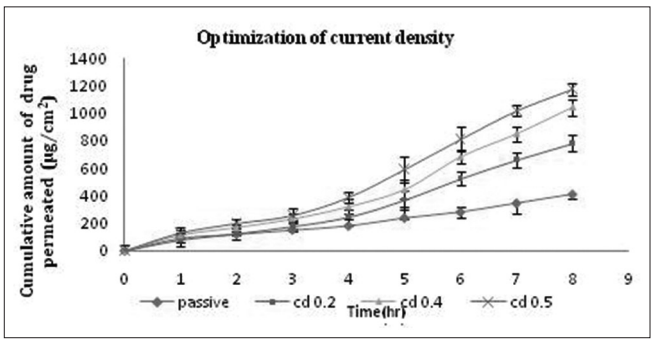

Figure 2: Effect of current density on permeation of risedronate sodium

higher ($P < 0.001$) for pulse ratio of 1:1 in comparison to higher pulse ratio. This may be because the skin remains in a polarized condition for more time at higher pulse ratio.

Effect of permeation enhancers

There was significant increase in the flux when Ethanol, and PEG-400 were used as penetration enhancers ($P < 0.001$). There was significant difference between flux of ethanol and PEG 400 as penetration enhancer ($P < 0.05$). Flux of 240.3 μg/cm^2/h obtained with combination of PEG 400 and iontophoresis [Figure 3]. This might be because of the compound was designed as a penetration enhancer for hydrophilic molecules. Its mechanism of action is based on increasing the water content of cell and altering the organization of the lipid structure of the stratum corneum, which increases the diffusion of the drug into the skin.

Synergistic effect of electroporation

In this study, electroporation pulses were given at interval of 1 min and followed by iontophoresis (0.5 mA/cm^2 ON: OFF ratio 1:1). The permeation of the drug was significantly increased after applying electroporetic pulses. After giving pulse of 10 ms at 110V the permeation was found to be 2140.65 μg/cm^2, while it was 2439.60 μg/cm^2 after giving electroporetic pulse 100 ms at 110V. At the pulses of 10 ms and 100 ms of 220V the permeation was found to be 2689.82 μg/cm^2 and 2811.90 μg/cm^2 respectively. The steady state flux values obtained were compared by mean of the one-way ANOVA, followed by Tukey-Kramer test for multiple comparisons [Table 2].

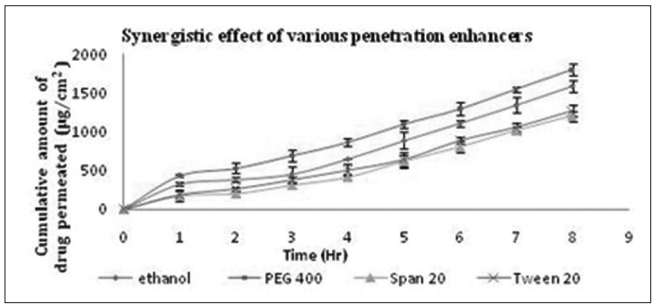

Figure 3: Synergistic effect of various penetration enhancers

There was significant increase in the flux when electroporation pulses were applied followed by iontophoresis (0.5 mA/cm^2 ON:OFF ratio 1:1) ($P < 0.001$). The flux 393.35 μg/cm^2/h was obtained when pulse of 100 ms − 220V was given, while it was 287.16 after giving pulse of 10 ms − 110V. The flux 301.82 μg/cm^2/h was obtained after giving electroporation pulses of 100 ms, 110V and flux 337 μg/cm^2/h was obtained at 10 ms at 220V. There was highly significant difference between flux of ionto gel and flux, followed by electroporation ($P < 0.001$). The disruption of the stratum corneum can dramatically influence overall skin permeability and it has been suggested that electroporation of its intercellular lipid bilayers might enhance percutaneous drug delivery. The biological composition and structure of the stratum corneum, the outermost layer of the skin, make it particularly attractive for electroporation.[22,23] The stratum corneum contains approximately 100 bilayer membranes in series, and electrical breakdown associated with dramatic increase in permeation. New aqueous pathways would be created within the stratum corneum due to electroporation of its lipid bilayers.[24,25]

The rationale for the combination of iontophoresis and electroporation was based on the difference between the mechanisms of action of these enhancers. Specifically, electroporation may disorder the lipid bilayers of the skin and create new transport pathways into the skin, thus facilitating

Table 1: Effect of pulsatile current on various permeation parameter

Permeation study	Q_8, (μg/cm^2)	Jss, (μg/cm^2/h)	Kp, (cm/h)	ER
Passive	412.10±37.08[a]	49.22±5.35[a]	1.64±0.17	1
Continuous CD	1177.16±85.4	194.55±14.8[ab]	6.48±0.79	3.95
CD 0.5 1:1	1289.72±99.85[a]	218.26±20.83[a]	7.27±0.82	4.43
CD 0.5 1:2	1221.41±96.00[b]	193.19±17.82[b]	6.43±0.904	3.92
CD 0.5 1:4	1092.19±92.1[b]	144.93±12.06[b]	4.83±1.33	2.94

[a]statistically significant difference from continuous current (P<0.001), [b]Statistically not significant difference from continuous current (P>0.05). Jss: Steady state flux, Kp: Permeability coefficient, Er: Enhancement ratio, Q_8: Cumulative amount permeated in 8 h, CD: Current density

Table 2: Comparative *In-vitro* permeation parameters of RS through human epidermis

Permeation study	Q_8, (μg/cm^2)	Jss, (μg/cm^2/h)	Kp, (cm/h)	ER	Lag time
Passive	286.65±25.78	32.41±4.98[a]	1.08±0.14	1	1.8 h
Iontophoresis 0.5 mA/cm^2 continuous current	483.15±12.6	52.6±3.2[a]	1.08±0.14	1.86	1.5 h
Iontophoresis 0.5 mA/cm^2 pulse current ON: OFF ratio of 1:1	950.38±84.3	148.23±13.35[a]	4.94±0.38	3.31	1.5 h
With PEG 400 (5% w/w) as penetration enhancer	1801.67±125.1	240.3±21.21[a]	8.01±0.55	6.28	1.2 h
Electroporation with 220V for 100 ms followed by pulsed iontophoresis	2811.90±182.87	393.35±26.39[a]	13.11±0.24	9.80	0.2 h

[a]Statistically significant difference (P<0.001). RS: Risedronate sodium, Jss: Steady state flux, Kp: Permeability coefficient, Er: Enhancement ratio, Q_8: Cumulative amount permeated in 8 h, PEG: Polyethylene glycol

passage of current during subsequent iontophoresis and resulting in increased transdermal transport.[26] Transport of most drugs across the skin is very slow and lag-times to reach steady state fluxes are in hours. The lag time was shortened drastically when iontophoresis was coupled with electroporation. Electroporation followed by iontophoresis led to a quick input and high flux.

Bommannan *et al*. reported the similar synergistic effect of iontophoresis and electroporation on transdermal delivery of luteinizing-hormone-releasing hormone (LHRH) *in-vitro*. Application of a single electroporation pulse prior to constant intensity iontophoresis consistently yielded 5–10-fold higher fluxes. The increased efficiency of electroporation + iontophoresis was attributed to the reduced impedance and size-selectivity of the skin.[27]

In-vivo permeation studies

The results of *in-vitro* permeation studies clearly indicated that iontophoresis, followed by electroporation caused 9-fold enhancement in the transdermal permeation of RS. The lag time for the permeation was significantly reduced.

The Pharmacokinetic data obtained was compared by means of the one way ANOVA followed by Turkey-Kramer test for comparison amongst different groups.

In the *in-vivo* studies in hairless rats significantly ($P < 0.001$) higher plasma concentration of RS was detected in Group-II treated with electroporation of 10 pulses of 220V of 100 ms at interval of 1 min followed by iontophoresis at current density 0.5 μg/cm^2 with ON:OFF ratio 1:1 than passive studies [Table 3].

There was more than 8 fold increase in the cumulative amount of drug permeated against passive permeation studies. In Group-II Significantly high C_{max} and low t_{max} signified that rate of permeation was very high and increased area under the curve suggested high extent of permeation. The relative bio-availability was 4.6 when calculated in comparison to passive studies. The results of *in-vivo* studies were completely in compliance with *in-vitro* studies.

Table 3: *In-vivo* (Pharmacokinetic) parameters after passive and iontophoretic study

Pharmacokinetic parameters	Passive permeation (group-I)	Iontophoresis followed by electroporation (group-II)
$AUC_{0\rightarrow 24}$ (μg^{-h}/ml)	30.962±5.2	121.511±8.2[a]
$AUC_{0\rightarrow \infty}$ (μg^{-h}/ml)	34.127±4.1	160.62±9.4[a]
C_{max} (μg/ml)	1.2921±0.58	6.942±0.43[a]
T_{max} (h)	3.10	1.1[a]
Lag time (h)	2.00	0.3[a]
Q_8,(μg/cm^2)	274.0±12.0	2312.0±102.0[a]
Relative bioavailability	1.00	4.60

All values are mean±SD, n=3, [a]Statistically significant difference from passive (P<0.001). Q_8: Cumulative amount permeated in 8 h, SD: Standard deviation

CONCLUSION

Permeation of RS was significantly improved from gel based iontophoretic delivery system when it was used in combination with other enhancement technique like electroporation and chemical penetration enhancers. Finally, it can be concluded that iontophoresis is a promising delivery system to obviate several unattractive features of RS oral administration. The permeation of RS was found to be increased when electroporetic pulses of 100 ms at 220V was given and followed by iontophoresis and it achieves the required flux when area of application is 1.55 cm.

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How to cite this article: Lahoti SR, Dalal S. Synergistic iontophoretic drug delivery of risedronate sodium in combination with electroporation and chemical penetration enhancer: *In-vitro* and *in-vivo* evaluation. Asian J Pharm 2015;9:171-7.

Source of Support: Nil. **Conflict of Interest:** None declared.