

Bosentan Monohydrate Vesicles Loaded Transdermal Drug Delivery System: *In Vitro In Vivo* Evaluation

Revathi M, Indira Muzib Y*

Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam (Women's University), Tirupati 517502, India

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ABSTRACT

This study elucidates the enhancement of the permeation of bosentan monohydrate through skin by encapsulating it in vesicles loaded transdermal delivery system. Niosomal vesicles were formulated by ether injection method. Formulation FN7 (span 60: cholesterol: poloxamer 401, 1.25:1:0.25) showed maximum entrapment efficiency of $96.7 \pm 0.037\%$ and was optimized for loading in to transdermal system. Transdermal systems were formulated using both hydrophilic and hydrophobic polymers like HPMC, HEC and EC. Formulation F1 with HPMC was optimized based on *in vitro* release ($99.21 \pm 1.45\%$) and was further evaluated for *ex-vivo* permeation. The results indicate that the *ex vivo* release ($98.13 \pm 1.65\%$) was as par with *in vitro* release and followed zero order super case-II transport mechanism. The *in vivo* studies were done on New Zealand male rabbits for oral and transdermal route. The results inferred no significant change in half-life of drug but a substantial difference in T_{max} , AUC and MRT was observed in transdermal systems. A two fold increase in AUC was observed in transdermal route ($18.609 \pm 7.251 \mu\text{g/ml/h}$) when compared to oral route ($9.644 \pm 5.621 \mu\text{g/ml/h}$). A controlled release was attained up to 35h and reservoir effect was observed and this may be due to the barrier properties of skin. Drug encapsulated niosomes were released in to the skin by loosening the lipid layers and the surfactant acted as penetration enhancer. The study infers that niosomes loaded transdermal patches of bosentan monohydrate can enhance the bioavailability and provided controlled release for better therapeutic efficacy and safety of drug.

Keywords: Bosentan monohydrate, niosomes, transdermal drug delivery, *ex vivo* permeation studies, *in vivo* kinetic studies.

INTRODUCTION

Transdermal drug delivery is the alternative route to oral drug delivery where drug can be directly administered in to the systemic circulation through skin¹. The main advantage of transdermal systems is first pass metabolism of a drug molecule can be avoided and controlled release of drug can be achieved².

Bosentan Monohydrate is a new generation endothelin receptor antagonist and is used to treat pulmonary arterial hypertension, which is a very progressive disease. It acts by blocking endothelin receptors there by reducing the constriction of pulmonary artery³. The characteristics like hepatic first pass metabolism, systemic bioavailability of 50%, half-life of 5 h⁴ and log P of 4.18⁵ have rationalized the drug molecule for its suitability to choose transdermal route⁶. It is also associated with many drug-drug interactions when taken orally like reducing absorption of warfarin⁷, contraception, immune suppression and is an inducer of cytochrome P450⁸. Despite the drug suits for transdermal delivery there is a limitation as the molecular weight of drug is 550 Daltons⁹. A new method for transdermal delivery of high molecular weight drug molecules was developed and patented by using a

polymer skin enhancer¹⁰. In this regard a new approach has been tried for facilitating the drug passage through stratum corneum and to increase the systemic bioavailability i.e., by encapsulating the drug molecule in surfactant vesicles and loading the vesicles in to transdermal film forming agents.

In this context niosomal vesicles were tried as they have the ability to entrap wide range of drug molecules^{11, 12} and high stability¹³. Formation of thermodynamically stable vesicles requires proper mixture of charge inducing agents and surfactants¹⁴. The encapsulation efficiency and stability of niosomes is influenced by HLB value of surfactants. Any surfactant with HLB value between 4 and 8 is ideal for a vesicle formation¹⁵. In niosomes passage of the drug through stratum corneum is modified by loosening the lipid layers, the surfactant acts as a drug carrier in to the systemic circulation¹⁶. A lot of work has been carried out in case of niosomes and was proved that niosomes can enhance topical delivery and are stable^{17, 18}. In the present study a new approach has been tried for enhancing transdermal delivery by using niosomes loaded transdermal patch¹⁹ of bosentan monohydrate without using any penetration enhancer, where the drug was

*Author for Correspondence: yindira1415@gmail.com

formulated in to niosomes and loaded into the transdermal patch. Non-ionic surfactants were chosen for the present work as they are having least irritable property²⁰. Niosomes were formulated using span 60, poloxamer 407 and cholesterol. Niosomes with high entrapment efficiency were selected for the formulation of transdermal patch using simple film forming agents like hydroxyl propyl methyl cellulose and hydroxyl ethyl cellulose. Formulated transdermal patches were evaluated for pharmacotechnical properties, *in vitro* studies, *ex vivo* studies, stability studies, skin irritation test and *in vivo* pharmacokinetic studies.

MATERIALS AND METHODS

Materials

Bosentan Monohydrate was received as gift sample from MSN Laboratories, India. Span 60, Poloxamer 407, Cholesterol, Di ethyl ether, Hydroxy Propyl Methyl Cellulose (HPMC), Ethyl Cellulose (EC) and Hydroxy Ethyl Cellulose (HEC) were purchased from Hi-Media laboratories Mumbai. All the reagents and solvents used were of analytical grade.

Formulation of niosomes of bosentan monohydrate

Niosomes of bosentan monohydrate were prepared by ether injection method. Surfactants and cholesterol were added to 10ml of diethyl ether and methanol²¹. In another beaker phosphate buffer saline (PBS) (pH 7.4) 100 ml along with drug was taken and heated up to 60°C. Surfactant mixture was added to PBS solution by using 14 gauge needle syringe by maintaining the temperature at 60°C (Table 1). The rapid evaporation of ether due to variation in temperature resulted in formation of niosomal vesicle. The resultant solution was lyophilized to obtain the dry product and it took 48h for complete lyophilization, mannitol was used as cryoprotectant and the obtained niosomes were stored in a glass vial at 5°C for further used. Niosomes with different concentrations of surfactant and lipid were prepared by repetition of the above method.

Formulation of niosomes loaded transdermal patches of bosentan monohydrate

Transdermal patches containing niosomal vesicles of bosentan monohydrate were prepared by casting method. Film forming agent was added to solvent mixture of dichloromethane (DCM) and methanol (3:2), stirred for 1h. Then niosomal vesicles of bosentan monohydrate and plasticizer propylene glycol (30% of polymer concentration) were added simultaneously and stirred for another 30 min on a magnetic stirrer. The resultant matrix dispersion was coated on a glass mould, covered with funnel for uniform evaporation and dried at room temperature for 24 h. Later the dried patches were separated from mould and stored in a desiccator. In the entire formulation niosomal vesicles equivalent to 10 mg of drug were added (Table 2). All the transdermal patches were developed without using any penetration enhancer.

Evaluation of niosomes of bosentan monohydrate

Size analysis and zeta potential

Zeta potential and poly dispersity index were measured by using zetasizer 2000 (Malvern instrument, UK) by

dispersing then niosome particles in deionized water at 25°C in clear disposable zeta cells.

Entrapment efficiency

Entrapment efficiency was determined by using centrifugation method. Niosomal suspension was taken in to centrifuge tubes and centrifuged (Macro scientific works, New Delhi, India) at 4000 rpm for 1hr. The drug was assayed both in the supernatant and sediment. To the sediment 0.1% triton was added to lyse the vesicles and filtered through 0.22 µm nylon filter. In the supernatant the drug concentration was found by directly analyzing the sample. Required dilutions are prepared and analyzed in UV Spectrophotometer (UV 1800 Schimadzu double beam spectrophotometer) at 272 nm and the percent entrapped was calculated according to Equation (1)¹⁹.

$$\% \text{ Entrapment efficiency} = [W_a - (W_s + W_p)/W_a] (1)$$

Where, W_a is the amount of drug in the system, W_s is the amount of drug in supernatant and W_p is the amount of drug in sediment.

In-vitro release study of niosomes of bosentan monohydrate

The *in-vitro* release studies were determined by membrane diffusion technique²². Niosomal vesicles were suspended in 0.3ml of PBS in 10 ml glass tube with one end covered with previously soaked cellulose dialysis membrane (molecular cut off 12,000 – 14,000 Daltons). The glass tube was suspended in a beaker containing 250 ml of PBS solution as release media and was allowed to rotate at 50 rpm by maintaining temperature at 37±0.4 °C. Aliquots of 3ml was withdrawn at every 1h time interval up to 24h by maintaining sink conditions and analyzed by UV-spectrophotometer at 272 nm.

Evaluation of niosomes loaded transdermal patches of bosentan monohydrate

All the transdermal patches were evaluated for pharmacotechnical properties. Weight of three patches was noted and the percentage weight was calculated. Folding endurance was calculated by repeatedly folding the transdermal patch at the same place till it broke. Film thickness was measured by using screw guage at three different sites and the mean value was calculated. Flatness was calculated by measuring the length of the strip of a patch after drying.

Drug content

The patch of area 3.14cm² was added to 100ml of PBS. The medium was stirred in a magnetic stirrer for a period of 24 h. After 24 h the solution was filtered and required dilutions were made and absorbance was noted at 272 nm using UV-Visible spectrophotometer.

Percentage moisture content

Transdermal patches were weighed (W_i) and transferred in to a desiccator containing silica gel and weighed till constant weight was achieved (W_c)²³. Moisture content study was done by using the Equation (2).

$$\text{Moisture content (\%)} = (W_i - W_c) / W_c \times 100 \quad (2)$$

Moisture absorption studies

Transdermal patches were weighed and placed in a desiccator containing 100 ml of saturated sodium chloride (RH 75%)²⁴. After 3 days patches were taken out and

weighed. Percentage of moisture uptake was studied by substituting the values of final and initial weight according to Equation (3).

$$\text{Moisture absorbed (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100 \quad (3)$$

In-vitro diffusion studies

In-vitro diffusion studies for the niosomes induced transdermal patch of bosentan monohydrate were carried out using modified Franz-diffusion cell²⁵. The receptor compartment contains PBS of 30 ml and the donor compartment contains transdermal patch of area 3.14cm² placed on cellulose dialysis membrane with molecular weight cut off 8000-14,400 Da. The dialysis membrane was soaked previously in PBS over-night. The whole assembly was kept on a magnetic stirrer stirred at 50 rpm and temperature maintained at 37±0.5°C. Sample of 3 ml was withdrawn for every 1h up to 28h and the amount of drug releases analyzed spectrophotometrically at 272 nm.

Ex vivo skin permeation studies

Ex-vivo skin permeation studies for the optimized transdermal patch was carried out using modified Franz-diffusion cell on excised rat skin²⁶. The male albino rat of wistar strain was anaesthetized by using chloroform and hair was shaved on the abdominal side. Full thickness of skin was removed surgically and the adhering fat on the dermis side was removed with the help of scalpels and by cleaning with isopropyl alcohol. Epidermis was separated by heat separation technique by keeping the skin in water at 60°C for 1 min. Epidermis was carefully removed with the help of forceps cleaned with distilled water. The skin was stored at -20°C for further use and to be used within one week. The prepared skin was hydrated by keeping in PBS for over-night.

Transdermal Patch of area 3.14 cm² was placed on the stratum corneum facing upwards and the dermal side facing receptor compartment. The receptor phase contains PBS (30ml) and the whole assembly was kept on a magnetic stirrer at 50rpm and temperature maintained at 37±0.5°C. Sample of 1 ml was collected for every 1h up to 30 h and the sink conditions were maintained. The amount of drug releases was analyzed spectrophotometrically at 272nm.

Mechanical properties of the optimized formulation

Tensile strength for the optimized formulation was carried out using TX.TA plus texture analyzer (Stable Micro Systems, UK) at preset conditions of test speed 2 mm/sec, pre-test speed 1 mm/sec, pro-test speed 10 mm/sec, force 100 g and 5 kg load cell²⁷. Film strip of dimensions 4 cm² was held between the clamps and pulled by the upper clamp at the speed of pre-set conditions. The force required to break the film and the elongation length were measured. The tensile strength and elongation at break were calculated according to Equation (4) and (5).

$$\text{Tensile strength (kg/mm}^2\text{)} = \frac{\text{Breaking force (kg)}}{\text{Cross-sectional area of patch (mm}^2\text{)}} \quad (4)$$

$$\text{Elongation at break (\%)} = \frac{[\text{Increase in length at breaking point (mm)} - \text{Initial length (mm)}]}{\text{Initial length (mm)}} \times 100 \quad (5)$$

Drug-polymer compatibility studies

FT-IR spectroscopy

This study was carried to find out the compatibility between drug and polymer. Sample and KBr were taken in the ratio of 1:100 in a mortar and triturated. A small amount of triturate was taken into a pellet maker and was compressed at 10 kg/cm² to form a transparent pellet using a hydraulic press. The pellet was kept in a sample holder and scanned from 4000 cm⁻¹ to 400 cm⁻¹ in FT-IR spectrophotometer (Bruker model 65 with opus software).

Differential scanning calorimeter (DSC)

DSC studies of pure drug, polymer, lyophilized niosomes and transdermal patch were carried out using DSC (METTLER STAR° SW 8.10) at a heating rate of 10°C/min in the temperature range of 20-300°C in the atmosphere of nitrogen. Empty aluminum pans were used as reference standard. The system was calibrated with indium before the study.

These studies were carried out to check the compatibility of drug with the polymers.

SEM Studies

Scanning electron microscope (Model JSM 6360, Jeol Make, United Kingdom) studies were done to characterize the surface of the niosomes and niosomes loaded transdermal patches of bosentan monohydrate.

Stability studies

The accelerated stability testing for the optimized formulation was done according to the ICH guidelines²⁸ for a period of 6 months at the following conditions: 40±2°C temperature and 75±5% RH. Conformational studies were done for the drug content, pH and physical properties.

Skin irritation studies

Skin irritation study was performed on male albino rats with average weight of 180-200 gm. The dorsal surface of the rat hair was removed by surgical scissors. The surface was cleaned with rectified spirit and the formulated transdermal patches were adhered with the help of surgical tape. Male albino rats were divided in to four groups (n=6) group I serves as normal, for group II blank patch (with-out drug) was secured, for group III medicated patch (with drug) was secured and for group IV a standard irritant 0.8%v/v aqueous solution of formalin was applied. The patch was removed after 15 min and the skin was observed for any irritation and edema. The experiment was repeated at 1h, 12h, 24h and 48h up to 7 days. Finally, the observations were made by physical examination and ranked as per scale prescribed by Draize²⁹.

Estimation of bosentan monohydrate in rabbit plasma by RP-HPLC

Chromatography was performed with Waters2695 HPLC provided with high speed auto sampler, column oven, degasser and 2996 PDA detector with dual wave length UV-Visible detector operated at 273 nm with class Empower-2 software. ODS (C18 250 x 4.6, 5μ) analytical column was used. Mobile phase used was 0.1% ortho phosphoric acid and acetonitrile in the ratio of 20:80 v/v% and the pH was adjusted to 4.6 by using tri ethanol amine.

Extraction procedure of bosentan monohydrate in plasma

Table 1: Formulation of niosomes of bosentan monohydrate.

Formulation	FN1	FN2	FN3	FN4	FN5	FN6	FN7	FN8
Span 60	1	2	2.5	3	0.1	1	1.25	1.25
Cholesterol	1	1	1	1	0.5	1	1	1.25
Poloxamer 407	-	-	-	-	0.25	0.25	0.25	0.25

All the quantities are mentioned in percentages.

Table 2: Formulation of niosomes loaded transdermal patch of bosentan monohydrate.

Formulation	HPMC	HEC	EC	*Propylene glycol	DCM: Methanol
F1	6	-	-	30%	3:2
F2	5	-	1	30%	3:2
F3	4	-	2	30%	3:2
F4	3	-	3	30%	3:2
F5	-	6	-	30%	3:2
F6	-	5	1	30%	3:2
F7	-	4	2	30%	3:2
F8	-	3	3	30%	3:2

All the quantities are mentioned in percentages, * was expressed as percentage weight of polymer

Table 3: Entrapment efficiency, particle size and zeta potential of niosomes of bosentan monohydrate.

Formulation	% Entrapment	Efficiency	Particle size	Zeta potential
FN1	32.3±0.023		220.4±1.291	-40.4±1.342
FN2	41.4±0.124		219.4±1.513	-40.8±1.021
FN3	59.6±0.081		215.9±1.082	-41.3±1.075
FN4	56.7±0.056		210.2±1.325	-44.2±1.492
FN5	79.7±0.162		151.3±1.466	-38.4±1.251
FN6	85.4±0.134		149.4±1.222	-40.1±1.084
FN7	96.7±0.037		146.1±1.227	-35.6±1.651
FN8	91.2±0.071		151.3±1.265	-39.5±0.982

Values are expressed as Mean±SD, n=3.

Table 4: Pharmacotechnical properties of the niosomes loaded transdermal patches of bosentan monohydrate.

Formulation	Thickness (mm)	% Moisture Content	% Moisture absorption	% Flatness
F1	0.59±0.011	0.72±0.027	0.92±0.069	100±0.001
F2	0.57±0.024	0.69±0.031	0.84±0.035	99.6±0.021
F3	0.48±1.035	0.57±0.130	0.79±0.047	100±0.011
F4	0.50±1.083	0.47±0.078	0.73±0.012	99.7±0.011
F5	0.45±0.076	0.70±0.037	0.89±0.026	100±0.011
F6	0.44±0.093	0.63±0.071	0.77±0.011	100±0.031
F7	0.42±0.032	0.51±0.136	0.68±0.014	100±0.011
F8	0.41±0.062	0.4±0.033	0.61±0.027	100±0.021

Values are expressed as Mean±SD, n=3.

Table 5: Pharmacotechnical properties of the niosomes loaded transdermal patches of bosentan monohydrate.

Formulation	Folding endurance	% Weight variation	% Drug content
F1	172±1.122	1.13±0.022	98.3±0.032
F2	171±1.024	1.20±0.122	97.8±0.025
F3	177±1.065	1.29±0.045	99.6±0.136
F4	172±1.252	1.23±0.082	97.6±0.057
F5	170±1.186	1.28±0.076	99.0±0.182
F6	176±1.057	1.19±0.115	98.9±0.025
F7	172±1.083	1.16±0.167	98.5±0.133
F8	179±1.261	1.12±0.033	99.4±0.176

Values are expressed as Mean±SD, n=3.

250µl of plasma, 50µl of internal standard and 10µl bosentan were taken in a centrifuging tube with teflon lined cap and 2 ml of acetonitrile was added, cyclomixing was done for 15 min. Then vortexed for 2 min to extract

the drug in to organic layer and finally centrifuged for 3 min at 3,200rpm speed. After centrifugation 20µL of organic layer was filtered through 0.45µ membrane and injected into HPLC at a flow rate of 1.0ml/min.

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-35.6 mV	-0.000276 cm ² /Vs
2	--- mV	--- cm ² /Vs
3	--- mV	--- cm ² /Vs

Zeta Potential (Mean) : -35.6 mV
 Electrophoretic Mobility Mean : -0.000276 cm²/Vs

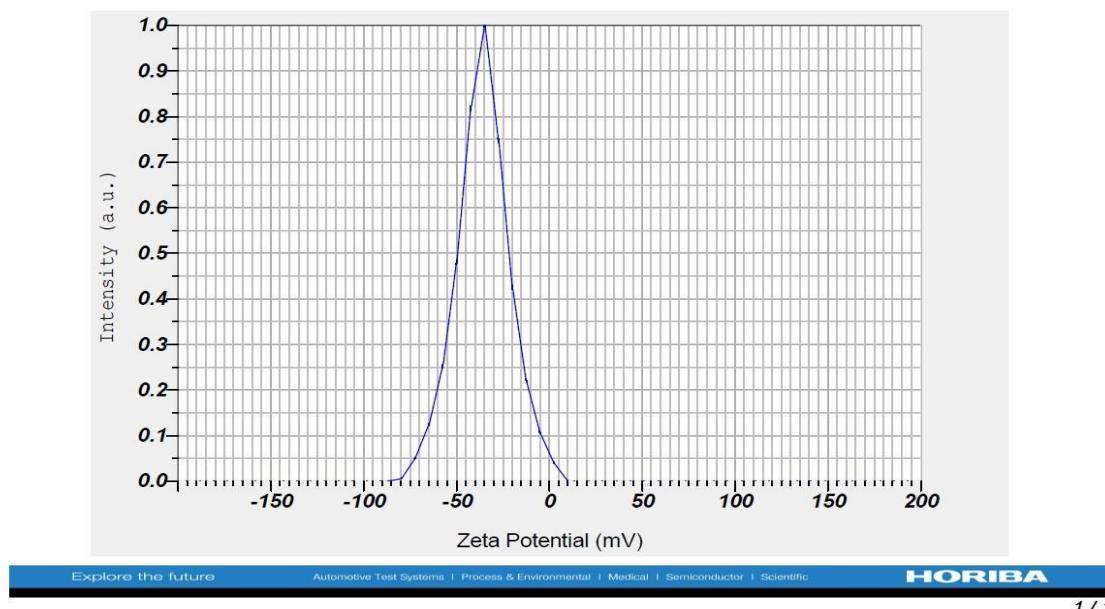


Figure 1: Zeta potential of niosomes (FN7) of bosentan monohydrate.

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	120.9 nm	10.7 nm	15.9 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	120.9 nm	10.7 nm	15.9 nm

Cumulant Operations

Z-Average : 1229.6 nm
 PI : 0.122

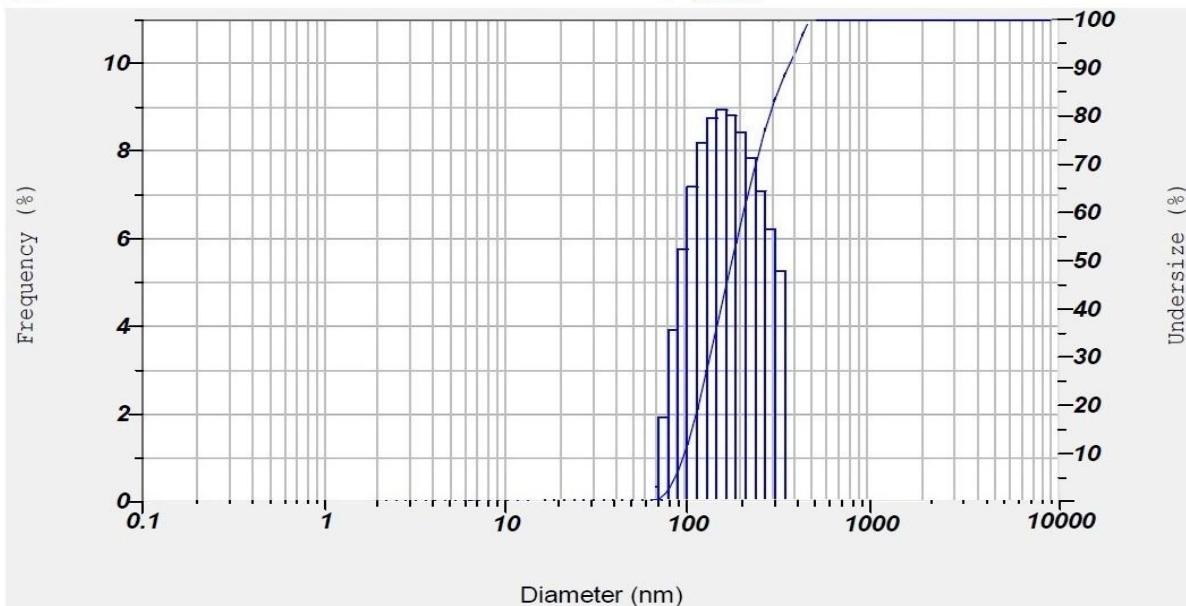


Figure 2: Particle size of niosomes (FN7) of bosentan monohydrate.

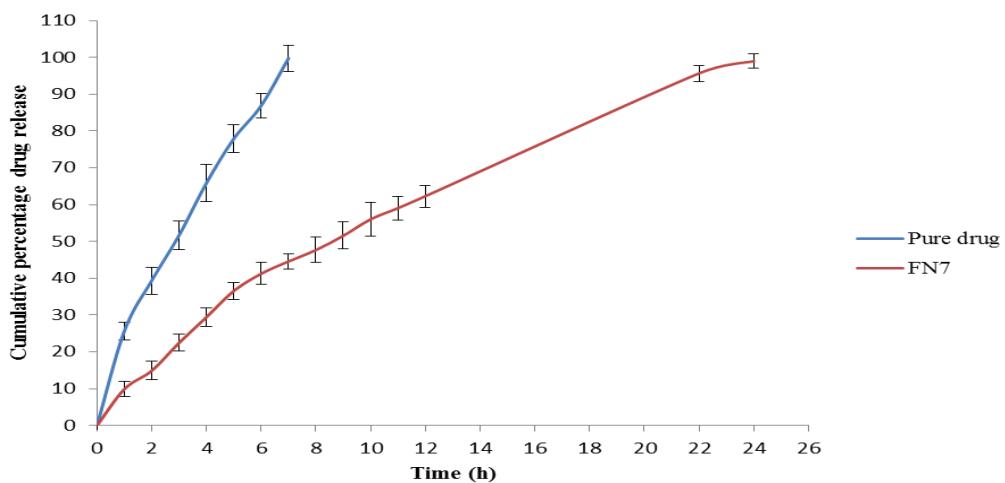


Figure 3: Comparative *in-vitro* release studies of niosomes of bosentan monohydrate (FN7) and pure drug in phosphate buffer saline media (Mean \pm SD, n=3).

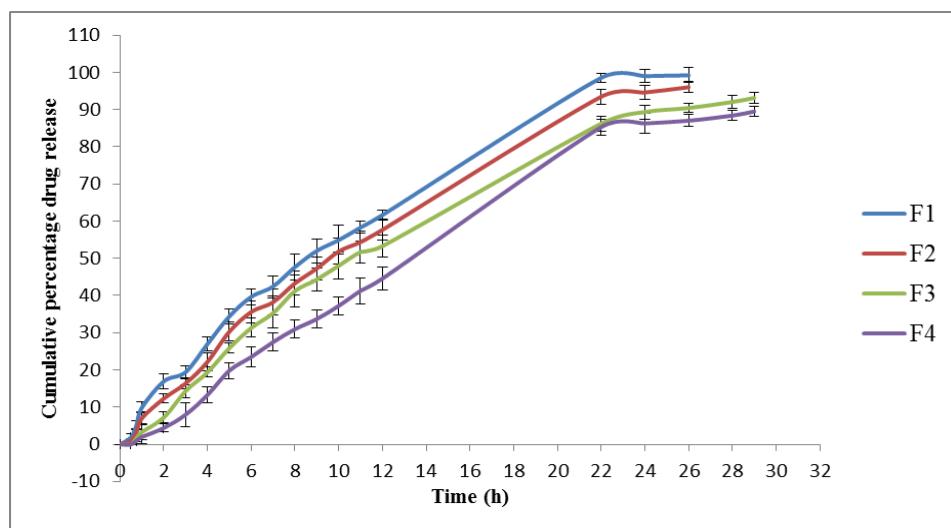


Figure 4: *In vitro* drug release profile of niosomes loaded transdermal patch of bosentan monohydrate in phosphate buffer saline media (Mean \pm SD, n=3) F1-F4.

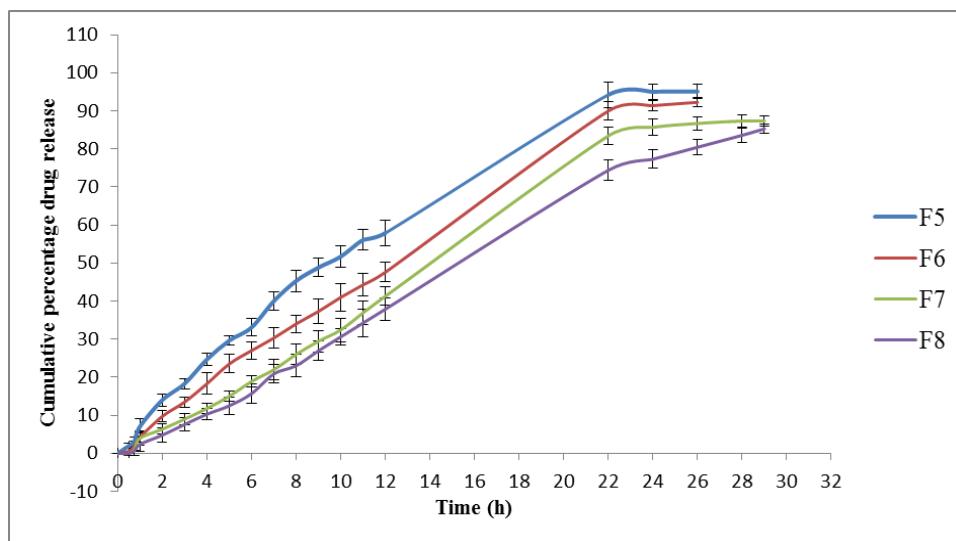


Figure 5: *In vitro* drug release profile of niosomes loaded transdermal patch of bosentan monohydrate in phosphate buffer saline media (Mean \pm SD, n=3) F5-F8.

Formulation	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$)	Permeability coefficient ($\text{cm}^2/\text{h}\times 10^2$)	Lag time (h)	Diffusion coefficient
F1	62.385 \pm 0.120	12.477 \pm 0.015	2.48 \pm 0.028	0.413 \pm 0.002
F2	61.175 \pm 0.109	12.235 \pm 0.086	1.78 \pm 0.019	0.296 \pm 0.001
F3	53.292 \pm 0.092	10.658 \pm 0.011	1.95 \pm 0.030	0.325 \pm 0.018
F4	53.366 \pm 0.086	10.673 \pm 0.010	0.38 \pm 0.017	0.063 \pm 0.011
F5	60.764 \pm 0.057	12.152 \pm 0.014	2.02 \pm 0.024	0.336 \pm 0.027
F6	59.631 \pm 0.021	11.926 \pm 0.017	0.05 \pm 0.016	0.008 \pm 0.003
F7	52.942 \pm 0.049	10.588 \pm 0.031	0.12 \pm 0.011	0.020 \pm 0.021
F8	50.073 \pm 0.022	10.014 \pm 0.023	0.42 \pm 0.017	0.070 \pm 0.037

Values are expressed as Mean \pm SD, n=3Table 6: *In-vitro* release studies of niosomes loaded transdermal patch of bosentan monohydrate.

Formulation	Zero order		First order		Higuchi	Korsmeyer-peppas
	R ²	R ²	R ²	R ²	n	
F1	0.9591 \pm 0.003	0.9177 \pm 0.018	0.9814 \pm 0.011	0.9546 \pm 0.012	0.8947 \pm 0.002	
F2	0.9681 \pm 0.001	0.957 \pm 0.001	0.9750 \pm 0.041	0.9448 \pm 0.004	1.0366 \pm 0.001	
F3	0.9612 \pm 0.019	0.9880 \pm 0.021	0.9788 \pm 0.032	0.9615 \pm 0.003	1.0392 \pm 0.001	
F4	0.9830 \pm 0.014	0.9723 \pm 0.035	0.9511 \pm 0.028	0.9736 \pm 0.026	1.1960 \pm 0.003	
F5	0.9646 \pm 0.018	0.9557 \pm 0.016	0.9771 \pm 0.026	0.9725 \pm 0.011	0.9489 \pm 0.003	
F6	0.9913 \pm 0.011	0.9433 \pm 0.019	0.9453 \pm 0.017	0.9538 \pm 0.016	1.1483 \pm 0.002	
F7	0.9869 \pm 0.023	0.9623 \pm 0.021	0.9290 \pm 0.037	0.9768 \pm 0.021	1.0895 \pm 0.001	
F8	0.9946 \pm 0.016	0.9740 \pm 0.027	0.9292 \pm 0.048	0.9801 \pm 0.032	1.1972 \pm 0.004	

Values are expressed as Mean \pm SD, n=3Table 7: *In vitro* release kinetics of niosomes loaded transdermal patch of bosentan monohydrate.

Bosentan Monohydrate	Physical Mixture		
Wave Number (cm ⁻¹)	Functional Group	Wave Number (cm ⁻¹)	Functional Group
3627	O-H	3628	O-H
3438	N-H Stretch	3436	N-H Stretch
2959	C-H	2956	C-H
1585	N-H Bend	1586	N-H Bend
1343	S=O	1342	S=O

Table 8: Comparison of the FT-IR spectrum of pure drug and physical mixture.

Parameters	0 Month	1 st Month	3 rd Month	6 th Month
Drug Content	98.3 \pm 0.032%	96.9 \pm 0.585%	95.28 \pm 0.391%	94.89 \pm 0.321%
Surface pH	6.8 \pm 0.011	6.2 \pm 0.013	6.4 \pm 0.001	6.4 \pm 0.003
Visual Inspection	Complies	Complies	Complies	Complies
Physical appearance	Acceptable	No change	No change	No change

Values are expressed as Mean \pm SD, n=3

Table 10: Skin Irritation Studies of niosomes loaded transdermal patch of bosentan monohydrate.

S.No	Formulation code	Erythema	Edema
1	Control patch	0 \pm 0	0 \pm 0
2	Medicated patch (F1)	1.25 \pm 0.221**	1.15 \pm 0.121*
3	Formalin induced group (0.8% v/v)	4.21 \pm 0.045	4.75 \pm 0.141

**p < 0.001 was found to be significant. Data was expressed as Mean \pm SD, n=6.

Erythema scale: 0, none; 1, slight; 2, well defined; 3, moderate; 4, scar formation.

Edema scale: 0, none; 1, slight; 2, well defined; 3, moderate; 4, severe.

In - vivo Pharmacokinetic studies

Studies were conducted according to ethical committee

No.:1677/PO/Re/S/2012/CPCSEA). *In vivo* pharmacokinetic studies for the optimized formulation were carried out by using male New Zealand rabbits of weight 1.3-1.5kg. Animals were kept under stabilized condition and provided with standard food and kept for fasting for 24 h before commencing the study. Rabbits were divided in to three groups (n=5) group-I received vehicle used to reconstitute the drug, for group-II bosentan monohydrate was given by oral route (11mg/kg), for group-III formulated niosomes loaded transdermal patch of bosentan monohydrate was adhered to the skin. Hair on the sides of vertebral column of the rabbit was removed and transdermal patch was adhered with the help of a surgical adhesive tape. Blood sample

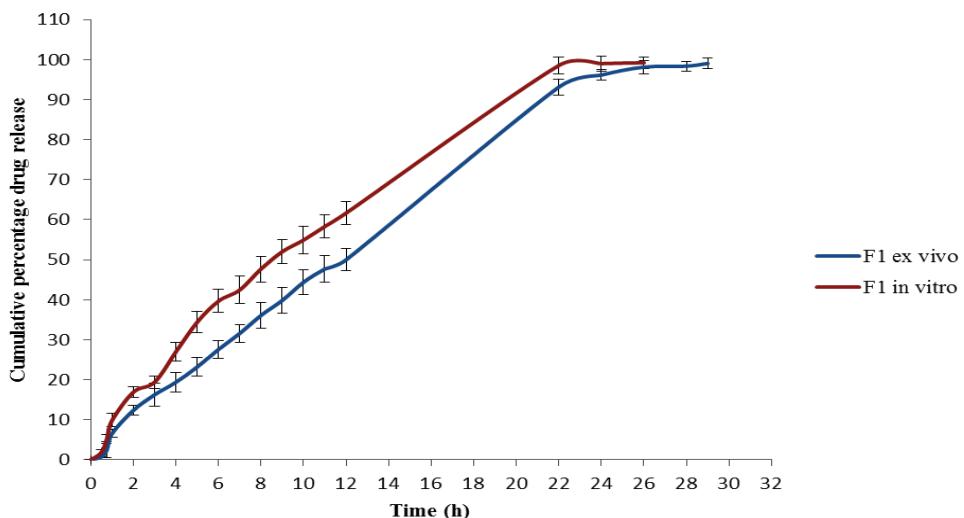


Figure 6: *In vitro* and *ex vivo* comparison studies of F1 niosomes loaded transdermal patch of bosentan monohydrate in phosphate buffer saline media (Mean \pm SD, n=3).

TA.XT^{plus}
Texture Analyser

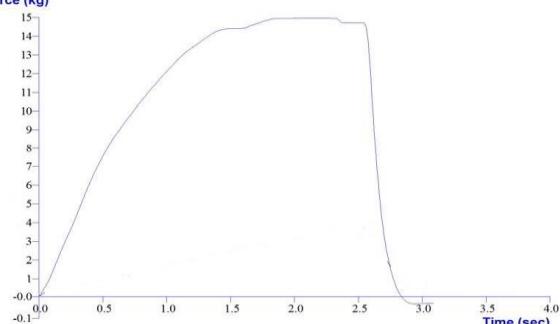
Stable Micro Systems

TA.HD^{plus}
Texture Analyser

Project Title: Untitled
TEXTURE ANALYSIS REPORT

T.A SETTINGS & PARAMETERS

Sequence Title: 1 RETURN TO START
Test Mode: Tension
Pre-Test Speed: 1.0 mm/sec
Test Speed: 2.0 mm/sec
Post-Test Speed: 10.0 mm/sec
Target Mode: Distance
Force: 100.0 g
Distance: 5.0 mm
Strain: 10.0 %
Trigger Type: Auto (Force)
Trigger Force: 5.0 g
Probe: A/TG ; TENSILE GRIPS
Batch: sample 7
Points per second: 200
Test Run by: system2



NOTES

This space is to enter notes regarding the test data.

Figure 7: Tensile strength of niosomes loaded transdermal patch of bosentan monohydrate (F1).

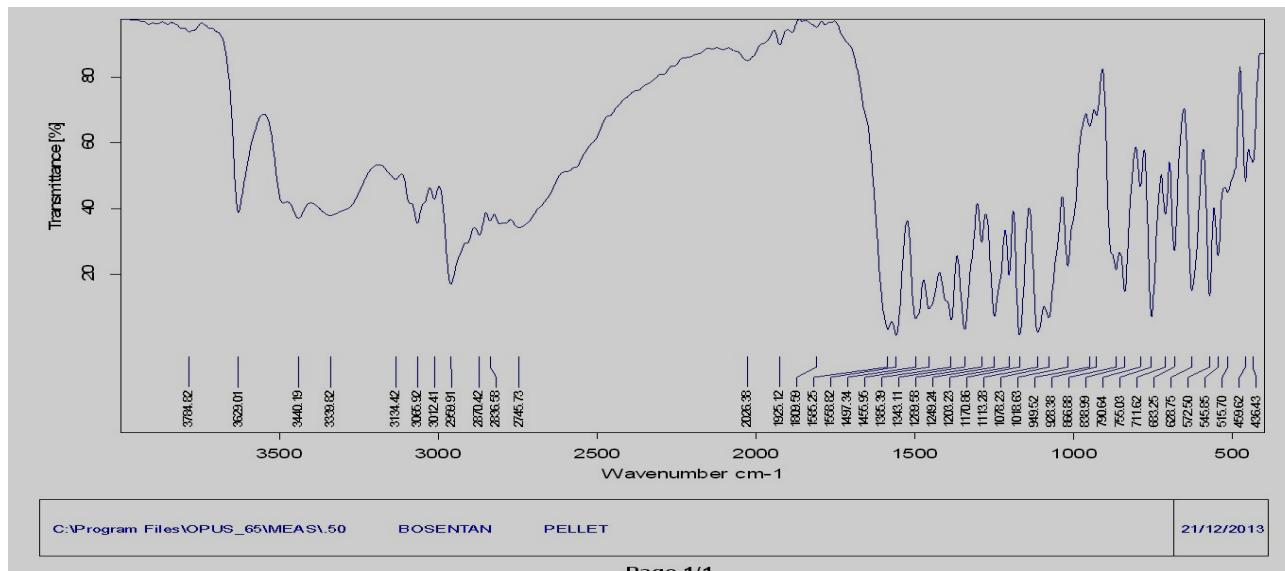


Figure 8: FT-IR Spectra of bosentan monohydrate.

of 1 ml was withdrawn from left marginal ear vein at time intervals of 0, 15 min, 30 min, 1h, 2, 4, 8, 12, 20 and 24h for oral and 0 min, 15 min, 30 min, 1h, 2, 4, 6, 10, 15, 20, 25, 30, 35, 40 and 45h for transdermal patch. The syringe used to draw blood was heparinized before using and plasma was separated immediately and stored in heparinized eppendorffs and frozen at -20°C till analyzed further to estimate *in vivo* parameters. Pharmacokinetic parameters were estimated from mean plasma concentration *vs* time graph. Pharmacokinetic parameters like C_{max} , T_{max} , half-life ($t_{1/2}$), area under curve (AUC) and mean residence time (MRT) were calculated. Two compartmental analysis was followed for transdermal patch³⁰ and one compartment for oral route.

Statistical Analysis

Statistical analysis was done using Microsoft excel. Paired t-test was used to compare the data, statistically significant difference was considered at $p<0.05$ and at $p<0.001$.

RESULTS AND DISCUSSION

Bosentan monohydrate loaded niosomes were successfully prepared by ether injection method using different ratios of span 60, poloxamer 407 and cholesterol. Entrapment efficiency was found to be in the range of $32.3\pm0.023\%$ to $96.7\pm0.037\%$ and the results were reported in Table 3. It has been reported previously that the surfactant with high phase transition temperature, low HLB value and high alkyl chain length (span 60) will have greater entrapment efficiency³¹. But a mere increase in entrapment efficiency by span 60 has been observed which may be due to the low aqueous solubility of span 60. For further enhancing the solubility and entrapment efficiency combination of span 60 and poloxamer 407³² has been taken, which proved to be successful. The addition of poloxamer 407 has drastically increased the entrapment efficiency due to the increase in solubility of the span 60 where it can entrap the drug into the bilayer of the vesicles. On further increasing the concentration of span 60 and cholesterol no furtherance in entrapment efficiency was observed. The reason may be the concentration of surfactant more than a limit may disturb the vesicle bi layer membrane which leads to reduction in entrapment efficiency³³.

Table 11: Pharmacokinetic parameters obtained after oral and transdermal administration of bosentan monohydrate in New Zealand male rabbits.

Pharmacokinetic parameters	Oral	Transdermal route
C_{max} ($\mu\text{g}/\text{ml}$)	1.36 ± 0.106	0.77 ± 1.287
T_{max} (h)	2 ± 0.221	$10\pm0.457^*$
Elimination rate constant (k_e)	0.151 ± 0.912	0.123 ± 0.864
Half-life (h)	4.6 ± 0.402	5.61 ± 1.012
AUC ($0\text{-}\infty$) ($\mu\text{g}/\text{ml}/\text{h}$)	9.644 ± 5.621	$18.609\pm7.251^*$
AUMC ($\mu\text{g}/\text{ml}/\text{h}^2$)	70.365 ± 3.471	$355.159\pm5.612^*$
MRT (h)	7.39 ± 1.457	$19.08\pm2.618^*$

*statistical significant difference at $p<0.05$ Values are expressed as Mean \pm SD, n=5

Figure 1 and Figure 2 illustrates the zeta potential and particle size of niosomes. As seen in Table 3 the vesicle size was found to be in the range of $146.1\pm1.227\text{nm}$ - $220.4\pm1.291\text{nm}$ and zeta potential in the range of $-35.6\pm1.651\text{mV}$ to $-44.2\pm1.492\text{mV}$ was noticed. Not much difference in the vesicle size of niosomes has been observed with the increase in concentration of surfactant and stabilizer. From the preliminary data available niosomes with high entrapment efficiency (FN7) was chosen for further studies.

In vitro diffusion studies of FN7 niosomes of bosentan monohydrate

In *in-vitro* diffusion studies of niosomal formulation of bosentan monohydrate (FN7) a higher release rate of drug was observed in the initial hours which may be due to the presence of drug on the surface of the vesicle and the wetting property of poloxamer³⁴. As seen in Figure 3 by the end of 5thh niosomes showed a 35% of drug release and the release rate was gradually decreased and extended up to 24h and by the end of 24h $98.9\pm0.891\%$ of drug was released, whereas in case of pure drug complete release of drug was observed with in 7h. Increase in drug release in initial hours will help to maintain the therapeutic concentration of drug in the plasma³⁵.

Evaluation of niosomes loaded transdermal patches of bosentan monohydrate

Transdermal patches of niosomes (FN7) of bosentan monohydrate were prepared by solvent evaporation method using both hydrophilic and hydrophobic polymers. All the dried patches were observed physically and found to be translucent. In the preparation of transdermal patches the good rheological properties like viscosity and flow properties of the casting solution produced translucent and uniform surfaced transdermal patches with good physical properties as illustrated in Table 4 and Table 5. As discussed in Table 4 a decrease in thickness of the transdermal patch was observed with the decrease in hydrophilic polymer content and no significant difference in the folding endurance was observed.

In-vitro release studies of niosomes (FN7) loaded transdermal patch of bosentan monohydrate

In-vitro release studies of the niosomes (FN7) loaded transdermal patches of bosentan monohydrate were conducted by using modified Franz-diffusion cell using PBS as release media and the release profiles were illustrated in Figure 4 and Figure 5. *In vitro* release studies of formulated transdermal patches showed a consistent release and the release was extended for more than 24h. The flux of niosomes loaded transdermal patches was found to be in the range of $50.073\pm0.022\mu\text{g}/\text{cm}^2/\text{h}$ to $62.385\pm0.120\mu\text{g}/\text{cm}^2/\text{h}$ with a lag time ranging from $0.05\pm0.016\text{h}$ to $2.48\pm0.028\text{h}$ and the results were summarized in Table 6. The enhancement in flux was observed with the increase in hydrophilic polymer content in transdermal patch. Percentage cumulative drug release of $99.21\pm0.451\%$ to $87.01\pm1.637\%$ was found in formulations F1 to F4 where as in F5 to F8 $95.11\pm1.219\%$ to $80.46\pm2.113\%$ cumulative drug release was found. The addition of

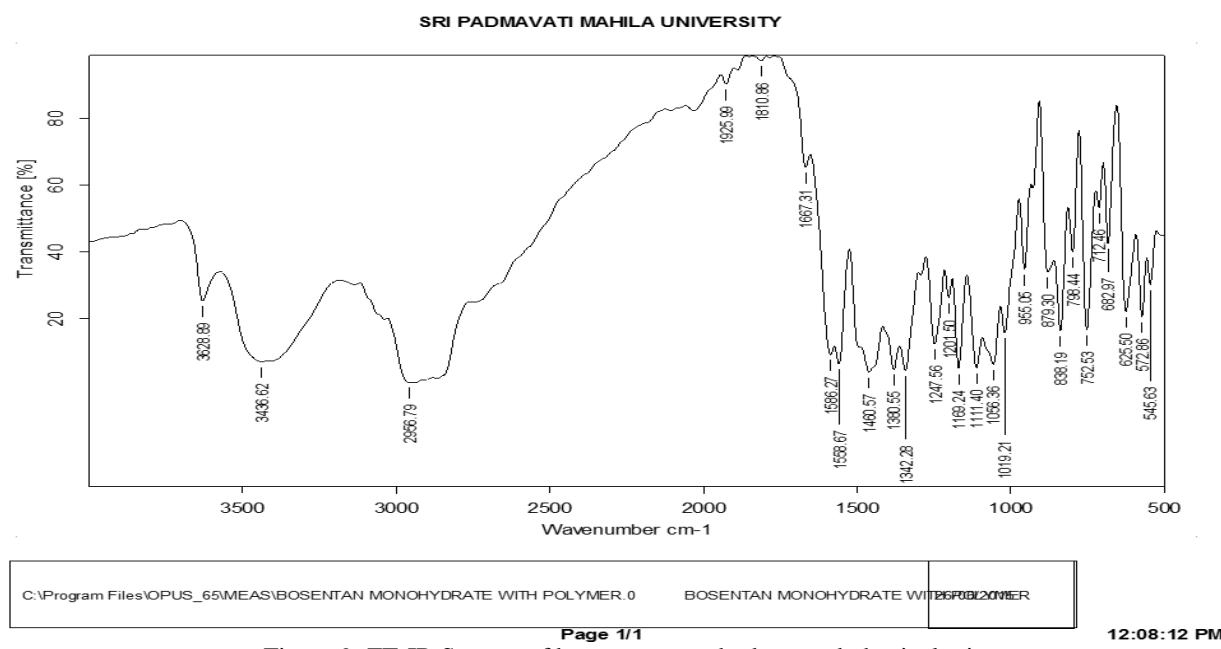


Figure 9: FT-IR Spectra of bosentan monohydrate and physical mixture.

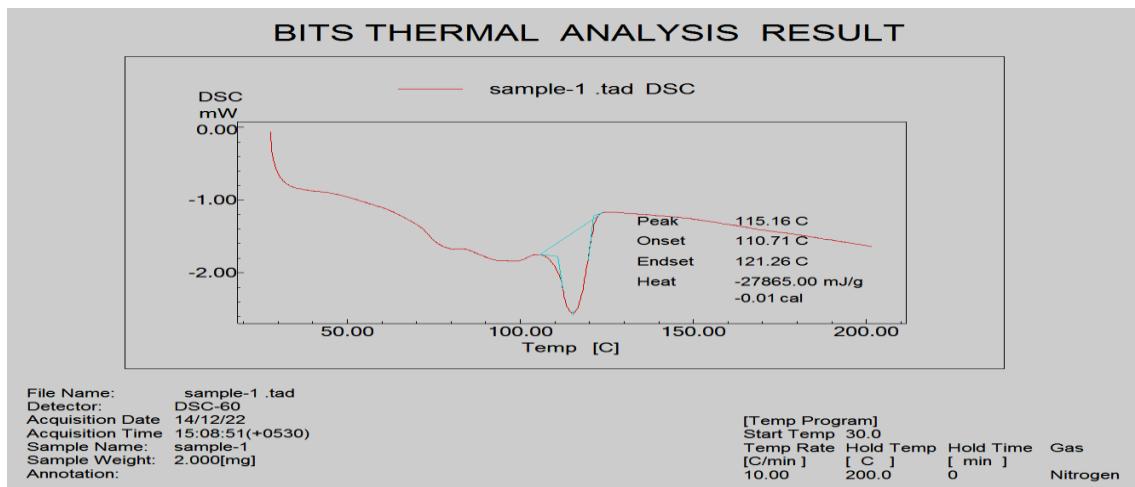


Figure 10: DSC Spectra of bosentan monohydrate.

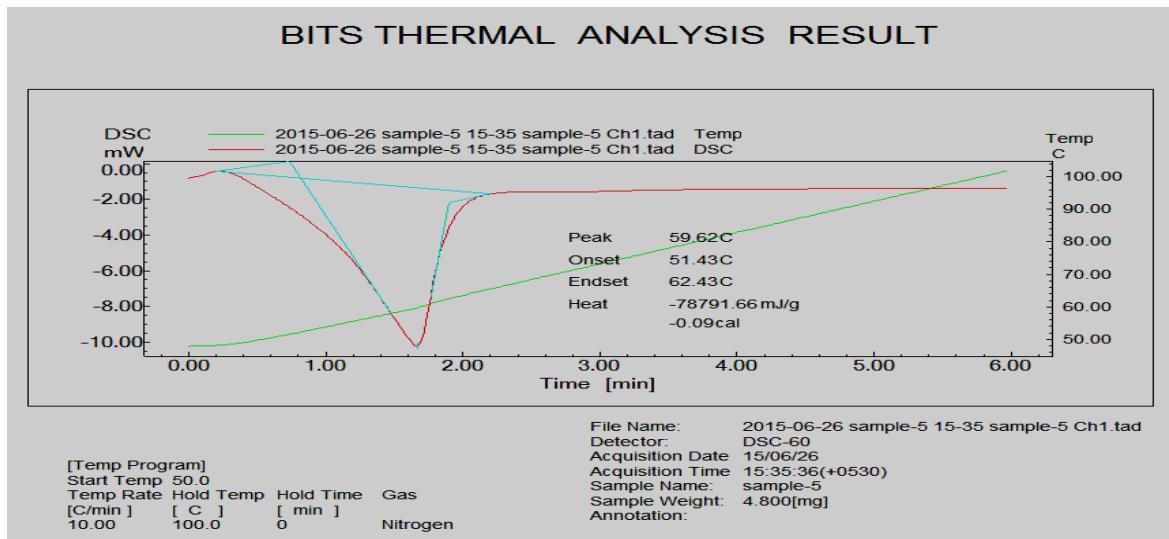


Figure 11: DSC Spectra of span-60.

hydrophobic polymer (EC) has prolonged the drug release by declining release rate. In transdermal patches with HEC (F5-F8) a declined release rate was observed

when compared to HPMC (F1-F4) and this can be attributed to the hydrophilic nature of HPMC compared to HEC. A variable percentage drug release was found in

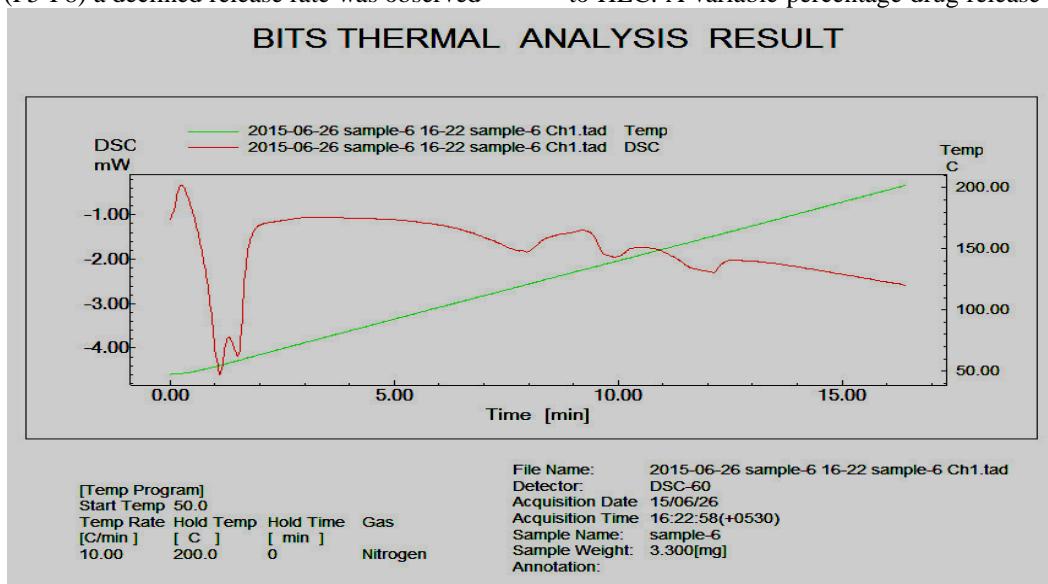


Figure 12: DSC Spectra of niosomes (FN7) of bosentan monohydrate.

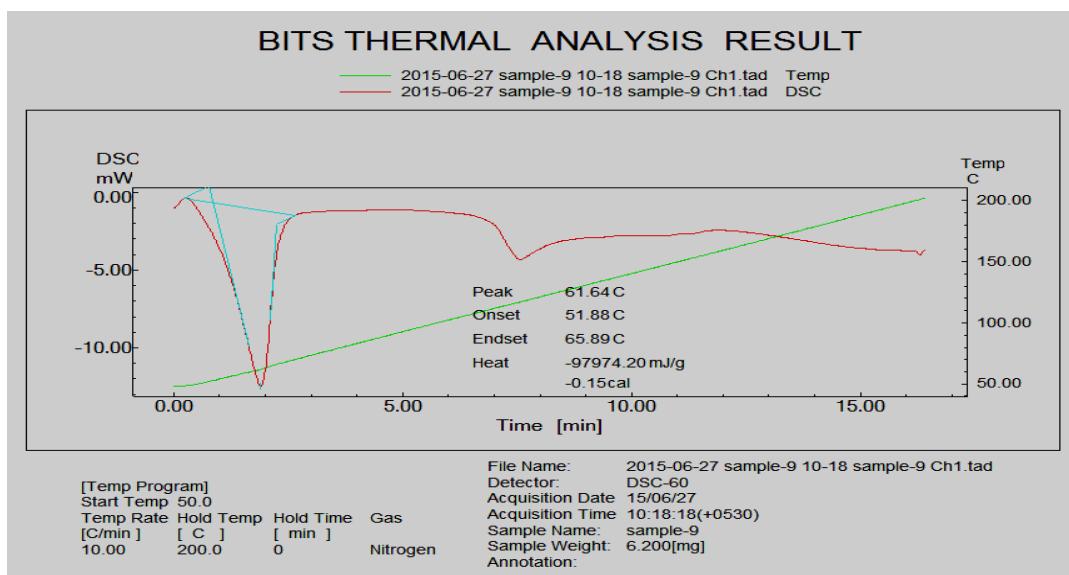


Figure 13: DSC spectra of niosomes loaded transdermal patch of Bosentan Monohydrate (F1).

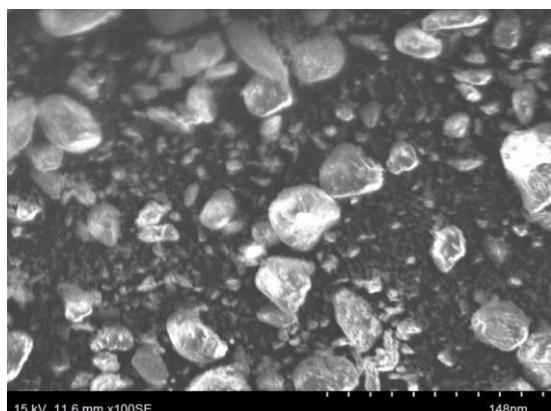


Figure 14: SEM image of niosomes (FN7) of bosentan monohydrate

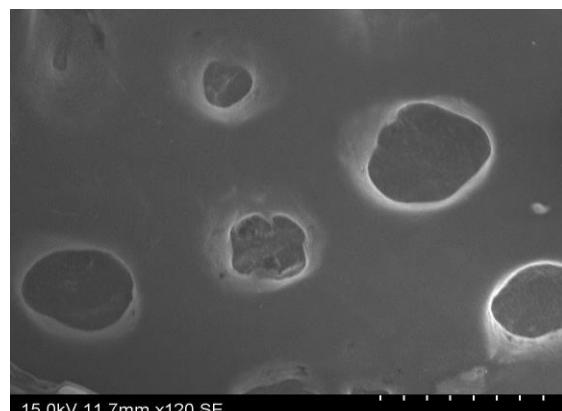


Figure 15: SEM image of niosomes loaded transdermal patch of bosentan monohydrate (F1).

all the transdermal patches with change in polymer concentration.

The dissolution profiles of transdermal patches of niosomes loaded bosentan monohydrate were hypothesized by applying different kinetics like zero order, first order and higuchi. Table 7 summarizes that the drug release is by zero order as it is having a linear regression compared to first order^{36,37}. A good linear regression in higuchi indicated that the drug release is by diffusion³⁸. To get more insight on kinetics of drug release mechanism korsmeyer-peppas equation was applied and n values of K-P model were in the range of 0.8947 ± 0.0020 . 7 to 1.1972 ± 0.004 showing that the drug release is controlled by super case-II transport in all the formulations^{39,40}.

Ex-vivo Permeation Studies

From the preliminary data available *ex-vivo* permeation studies for F1 were carried out as the *in vitro* drug release of F1 was found to be $99.21 \pm 1.45\%$ with a flux of $62.385 \pm 0.120 \mu\text{g}/\text{cm}^2/\text{h}$ by the end of 24h. As depicted in Figure 6 *ex vivo* permeation studies of F1 by the end of 24h drug release was found to be $98.13 \pm 1.65\%$ with a flux of $57.31 \pm 0.218 \mu\text{g}/\text{cm}^2/\text{h}$ and lag time of $1.39 \pm 0.01\text{h}$. No significant difference in drug release pattern was observed when compared to *in vitro* studies ($p < 0.05$).

It was reported that drugs with $\log P > 3$ ($\log P$ of bosentan monohydrate is 4.18) will have high diffusion in to the stratum corneum and less transport in to the systemic circulation i.e., release media⁴¹. But in case of niosomes loaded transdermal patches of bosentan monohydrate it was observed that *ex vivo* permeation studies were as par with the *in vitro* diffusion studies, the results summarizes that the transportation of drug in to release media has not changed when compared with *in vitro* studies. Interestingly it was observed that without using any penetration enhancer the flux values were maintained and no effect on drug release was observed. This can be concluded as an added advantage to this type of systems.

Mechanical properties of the optimized formulation

Mechanical properties of the optimized formulation were calculated according to Equation 4 and 5. Figure 7 illustrates the tensile strength of the HPMC loaded transdermal patch was found to be $0.375 \pm 0.089 \text{ kg/mm}^2$ with a percentage elongation at break value of $7.5 \pm 1.213\% \text{ mm}^2$.

Drug-polymer compatibility studies

The FT-IR spectrum of bosentan monohydrate and physical mixture of bosentan monohydrate with polymers was shown in Figure 8⁴² and Figure 9 respectively, no change in the prominent peaks was observed as illustrated in Table 8.

In DSC thermogram of bosentan monohydrate a sharp endothermic peak was observed at 115.16°C ⁴² and in span 60 endothermic peak at 59.62°C was observed as seen in Figure 10 and Figure 11 respectively.

Where as in case of DSC thermogram of niosomes endothermic peak at 49.28°C and in niosomes loaded transdermal patch endothermic peak at 61.64°C was observed in Figure 12 and Figure 13 respectively. As seen

in DSC thermogram of niosomes a complete encapsulation of drug in span 60 was observed. In case of niosomes loaded transdermal patch also the pure drug endothermic peak was not observed indicating no structural change in the niosomes after entrapping them in to transdermal patch. In both the cases the disappearance of endothermic peak of pure drug was observed.

Scanning Electron Microscopy (SEM)

As seen in Figure 14 niosomes appeared in spherical with sharp boundaries, no irregularity has been observed on the surface of the niosomes. SEM image of transdermal patch showed even surface and uniform distribution of the vesicles throughout the patch and it can be visualized in Figure 15.

Stability studies

The optimized formulation (F1) was subjected to stability studies at accelerated stability testing conditions as per ICH guidelines. Table 9 reveals that the stability testing data of drug content, surface pH, visual inspection and physical properties and it can be concluded that the transdermal patches were stable. The results of drug content show a little variation with a low standard deviation ($p < 0.05$).

Skin irritation studies

Table 10 summarizes the skin irritation studies of niosomes loaded transdermal patch of bosentan monohydrate and it was found that negligible erythema and edema was found in case of control patch and drug loaded patch. Significant difference from standard irritant ($p < 0.001$) was found and the patches were found to have very less erythema and edema which is in permissible limits.

In-vivo pharmacokinetic studies

In-vivo pharmacokinetic studies of orally administered and niosomes loaded transdermal formulation of bosentan monohydrate were estimated from mean plasma concentration *vs* time profile graph as shown in Figure 16.

Table 11 summarizes the pharmacokinetic parameters estimated from oral and transdermal route. The C_{\max} value in oral route was found to be $1.36 \pm 0.06 \mu\text{g}/\text{ml}$ at $T_{\max} 2 \pm 0.221\text{h}$, where as in transdermal route the C_{\max} value was $0.77 \pm 1.287 \mu\text{g}/\text{ml}$ at $T_{\max} 10 \pm 0.457\text{h}$. In the oral route the high C_{\max} and low T_{\max} values were due to rapid absorption of the drug where as in case of transdermal route the decrease in C_{\max} and increase in T_{\max} was observed which is due to the barrier properties of the skin. The elimination half-life was found to be $4.6 \pm 0.402\text{h}$ in oral route where as in transdermal it was found to be $5.61 \pm 1.012\text{h}$. No significant difference in half-life of the drug molecule was observed when compared to oral route. The AUC value in case of oral route was $9.644 \pm 5.621 \mu\text{g}/\text{ml}/\text{h}$ where as in case of transdermal delivery AUC was extended up to $18.690 \pm 7.251 \mu\text{g}/\text{ml}/\text{h}$ and the MRT values were found to be $7.39 \pm 1.457\text{h}$ for oral and $19.08 \pm 2.618\text{h}$ for transdermal route. A two fold increase in AUC values was observed in transdermal route which is due to the prolonged release and slow depletion of drug through the skin. Increase in AUC will result in increase in

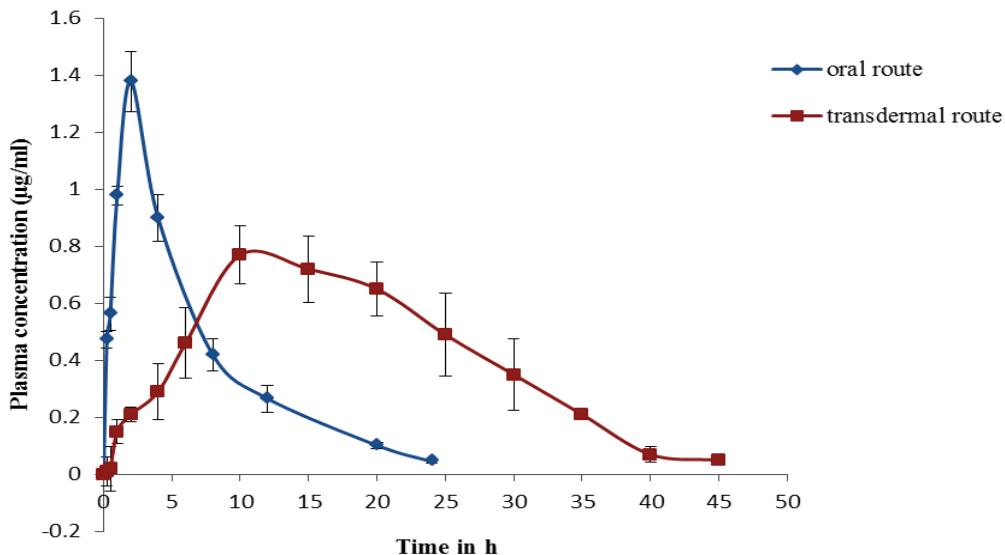


Figure 16: Mean plasma concentration vs time profile of oral route and transdermal route of bosentan monohydrate in New Zealand male rabbits (mean \pm SD, n=5).

bioavailability of drug, the high AUC values in transdermal route indicates the presence of drug for longer duration and this was supported by the MRT value of transdermal route where it was high compared to oral route. The multiplication of MRT values by 2.5 times in transdermal system indicates the presence of drug for longer duration thereby providing controlled delivery of drug in to the systemic circulation. The reservoir effect was observed in transdermal system as the drug release in to plasma was observed after removal of the patch up to 5h. Extravascular two compartmental analysis for transdermal route and one compartmental analysis for oral route was found to be best fit basing on the regression values.

The encapsulation of drug in to niosomes has alleviated its transdermal permeability by altering its lipophilicity and nano-sizing the drug molecule further supplemented the release of drug through the skin. The findings of *in vivo* suggest the potentiality of usage of niosomes loaded transdermal systems of bosentan monohydrate. The pharmacokinetic data of niosomes loaded transdermal delivery of bosentan monohydrate established the longer therapeutic action which provided a controlled release of drug for a better therapeutic activity and safe usage of drug compared to oral route.

CONCLUSIONS

In this research niosomes loaded transdermal patches of bosentan monohydrate were developed to facilitate the even passage of drug molecule (molecular weight is 550 Daltons) through stratum corneum. The anticipation of incorporating drug loaded niosomal vesicles in transdermal patches without using any penetration enhancer for passage of drug through skin was proved to be successful. The surfactant surrounding the drug molecule acted as synergist and facilitated the drug passage through stratum corneum in to the release medium. The results of *in vivo* kinetic studies depicted

the controlled release pattern of drug in to the systemic circulation. The current study showed a positive way of incorporation of niosomal vesicles of bosentan monohydrate in transdermal systems which provided safe and acceptable drug release kinetics.

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