11. Summary and Conclusion

11.1. Summary

11.1.1. Introduction

Arthritis is main reason of disability in human in many developed and developing countries. Arthritis can cause change in the shape or in fundamental material of joints by any modification or there may be detraction of support from joints. There are various types of arthritis that can be found in humans nearly around 200 types, however inflammatory and metabolic arthritis are found to have highest prevalence. Inflammatory arthritis and metabolic arthritis are also known as a rheumatoid and gouty arthritis respectively.

Rheumatoid arthritis (RA) is an autoimmune disease which mainly shows symptoms like systemic inflammation, autoantibodies and persistent synovitis. Moreover, RA patients also suffer from several other indications like weight loss, low grade fever, fatigue, dry mouth, dry eyes, lump formation under the skin in hands or elbows etc. In majority cases, RA involves pair of joints which accounts for limbs but can also involve more than one joint, like small joints present in wrists and hands. Eventually, various joints such as knees, shoulders, elbows, feet, and ankles can also be affected. It is considered to be an autoimmune disease, wherein our body's immune system misinterprets own cells as invaders. Immune system attacks the synovium and membrane lining in the joints, which leads to swelling and solidifying of the joint capsule. Further, it also affects underlying cartilage and bone. Being autoimmune disease immunosuppressants are prescribed for its treatment. They act by inhibiting the activation of T cell. Methotrexate and Tacrolimus (TAC) are both immunosuppressant which can be used to treat RA.

TAC is an immunosuppressant which is indicated in organ rejection. TAC ointment is also available which is indicated for topical autoimmune diseases such as psoriasis and eczema. Moreover, there are reports suggesting its therapeutic benefits in RA and it also has a better adverse effect profile when compared to other immunosuppressants. Thus, TAC is selected for the present project. TAC is available in the market in various dosage forms which includes, Tablet (Crolim-Ranbaxy

Laboratories), Capsule (Olmis Cap: Unichem Laboratories, Tacloram: Wockhardt (Biotech). Presently, oral dosage form is indicated for organ rejection and ointment is indicated for psoriasis. Tablets (1 mg, 2mg and 5mg) are administered depending on the requirement of the patient. Ointment (Olmis: Unichem Laboratories) is available in the market but drug absorption is incomplete. Oral bioavailability of TAC is 24%, Protein binding is ≥ 98.8%, Biological half-life is 11.3 hours for transplant patients (range 3.5-40.6 hr). It is primarily metabolized by the live microsomal enzyme and mostly excreted in faeces. Adverse effects like gastrointestinal disturbances such as diarrhea, nausea, vomiting, stomach pain etc. were caused by TAC and these adverse effects became severe in various cases which lead to the withdrawal of therapy. TAC has a limitation of poor oral bioavailability, food dependent absorption, gastrointestinal disturbances.

Gout can be defined as a condition of uric acid disturbance in the body. It is the most studied and scrutinized class of arthritis. When monosodium urate crystals (MSU) deposit in the tissues, it leads to the presentation of Gout. Uric acid crystals are formed when serum uric acid (SUA) increases over a specific limit and accounts for the main cause of deposition of uric acid crystals. Joint pain, joint stiffness, joint swelling and joint redness are the main symptoms of gout. Xanthine Oxidase inhibitors are the most prevalent and widely used treatment option for gout. As reported in humans, inhibiting xanthine oxidase results in the reduction of production of uric acid, and various drugs that inhibit xanthine oxidase are indicated for the management of hyperuricemia and other similar medical conditions including gout. Therefore, xanthine oxidase inhibitors qualify as potential candidates (e.g. Allopurinol and Febuxostat) and are always preferred for the treatment of chronic gout.

Febuxostat (FBX) is a new, highly potent, non-purine selective xanthine oxidase inhibitor that is responsible for the inhibition of both oxidized and reduced forms of xanthine oxidase. It has been reported that (FBX) when compared to allopurinol is more efficient in the reduction and maintenance of serum urate levels. FBX is marketed as a 40, 80 and 120 mg tablet to be administered once a day and are marketed under the names Fabulas, Feboxa, Febuget, Febucip etc. Bioavailability achieved after oral administration of FBX is 38% and experiences food interaction. Oral bioavailability of

FBX is reduced because of its low (< 15 µg/ml) aqueous solubility and extensive enzymatic degradation in intestine and liver. Furthermore, due to food interaction, FBX peak plasma concentration (C_{max}) in blood is reduced by 38–49%. Shortcomings that come with FBX are poor bioavailability, food affected absorption, gut-wall metabolism, gastrointestinal disturbances (nausea, diarrhea, stomach pain, ulcers, vomiting).

If we deliver these drugs by transdermal route instead of conventional oral route, then the above-mentioned problems associated with the marketed formulations of FBX and TAC can be overcome. Enhancement in bioavailability by avoiding first pass metabolism of a drug and prevention of the gastrointestinal disturbances can also be achieved However, the outermost skin layer, stratum corneum (SC), composed of dead keratinized tissue of about 10–20 µm in thickness constitutes a major obstacle and severely limits the delivery of molecules. A variety of physical and chemical methods have been explored to breach SC including use of penetration enhancers, lasers, electrical energy, ultrasound, radio frequency, thermal energy and microneedles.

With prolonged use, it is reported that chemical penetration enhancers cause skin toxicity and irritation. Thus, for present work, various nanocarriers were employed for enhancement of transdermal permeation. For example, Wei Lei et al. prepared transdermal gel loaded with transfersomes containing TAC and noted that transfersomes-gel loaded TAC has higher effective retentions in epidermis and dermis when compared to traditional-gel and commercial ointment. Moreover they established that TAC loaded transfersomes have enhanced penetration of drug in skin and enhanced therapeutic efficacy improvement by performing in-vivo study. Sanju Singh et al. prepared the niosomal gel of FBX and proved the transdermal potential and anti-gout efficacy of developed formulation.

“Cubosomes” are the “discrete, sub-micron, nanostructured particles of the bicontinuous cubic liquid crystalline phase. The term "bicontinuous" used in the definition of cubosomes describes two individual hydrophilic areas which are separated by the bilayer. The structure of bicontinuous cubic crystalline materials is reported to be suitable for controlled release formulation making it a popular area of research.

Cubosomes are colloidal dispersions having a size range of 100 to 300 nm which can be prepared by dispersing the bicontinuous cubic liquid crystalline structures in aqueous medium having surface active agents. Various classes of drugs like hydrophilic, lipophilic and amphiphilic drugs can be encapsulated by cubosomes.

Majority of the times, such nanocarriers are not able to sufficiently enhance the transdermal permeation and achieve desired plasma concentration, and there comes the need of use of some physical penetration enhancement techniques like microneedle (MN) patch. Application of microneedles proves to be advantageous as it facilitates the permeation across the toughest barrier, stratum corneum. Another advantage of microneedles is that, they are very short length and have narrow diameter, and so are incompetent to reach the nerve endings and are thus painless. Essential advantages of microneedles are: lack of pain, delivery of large molecules efficiently, patient compliance, avoidance of first pass metabolism, target based delivery, minimal potential for tissue trauma from an injection.

11.1.1.1. Aim of study

- Enhancement in permeation of drug(s)
- Sustained drug release for prolonged period
- Improvement of bioavailability
- Sidestepping gastro-intestinal disturbances associated with selected drugs
- Reduction in dose and dosing frequency
- Improvement in patience compliance
- Effective treatment or management of both types of arthritis

11.1.2. Analytical Method development

HPLC method of TAC was developed for the present research study using C_{18} column (4.6 µm X 150mm) at 220 nm λ_{max} and 60 °C column temperature. Then, calibration plot of TAC using the developed method was generated which was used for quantification of TAC in various samples except samples of Pharmacokinetic and ex-vivo studies. For Ex-vivo study, HPLC method was developed and calibration plot was obtained using C_{18} Thermo Scientific column and Agilent 1220 systems with UV

detector at λ_{max} of 220 nm. For pharmacokinetic study, LC-MS method was developed and calibration plot was obtained using C_{18} column and Sciex QTRAP® 4500.

In case of FBX, UV visible spectrophotometric method was developed and calibration plot for FBX was developed which was used for quantification of FBX in various samples except samples of entrapment efficiency of FBX in cubosomes, ex-vivo and pharmacokinetic studies. For estimation of FBX in cubosomes HPLC method of FBX was developed and calibration plot was prepared using C_{18} column and Agilent gradient HPLC. For estimation of FBX in samples of ex-vivo and pharmacokinetic studies bio-analytic method was developed using Agilent gradient HPLC and C_{18} column and calibration plot was prepared.

11.1.3. Preformulation studies

Authentication of drugs and evaluation of their compatibility with other formulation components of interest was done as a part of preformulation studies. TAC and FBX both were authenticated based on the comparison of their melting point, UV absorption spectra, FTIR spectra and DSC thermograms with that available in literatures. In case of TAC, authentication using UV absorption spectra was impossible due interference of organic solvent due to its wavelength cutoff value. Thus, authentication of TAC was done using FTIR and DSC. In FTIR, it's showed all characteristic peak and in DSC thermogram of received samples gave endothermic peak at 127.66 °C which is similar to its reported melting point which is 124-128 °C. Solubility study of TAC revealed that it has a maximum solubility in acetonitrile and good solubility in organic solvents like DMSO, ethanol, methanol, and lipids like Glyceryl Monooleate (GMO) and Glyceryl Monostearate (GMS). In case of FBX, it showed all characteristic peaks in UV absorption spectra at λmax of 315 nm. Moreover, all characteristic peaks were also observed in FTIR spectra of obtained sample of FBX. A DSC thermogram of FBX also showed characteristic endothermic peak at 207.4 ° which falls in the reported melting point range of FBX i.e. 209-212 °C. Solubility study of FBX suggested that it has good solubility in various organic solvents like DMF, DMSO, ethanol, methanol and in lipids like GMO and GMS.

11.1.4. TAC loaded cubosomes

To prepare the cubosomes of TAC bottom up approach was used. The cubosomes of TAC was optimized using QbD approach. During optimization of TAC loaded cubosomes, 2-level fractional factorial design was employed to screen out various Critical Quality Attributes (CQAs) which had significant impact on cubosomes of TAC. The CQAs which were screened out were particle size, particle size distribution and % entrapment efficiency. These CQAs were further optimized using 3^2 factorial design generated by Design Expert 7.0. An optimized batch was prepared using composition of Critical Material Attributes-CMAs (GMO and PVA) suggested by overlay plot of 3^2 factorial design generated by Design Expert 7.0. Various characterization test of this optimized batch was performed like % entrapment efficiency, vesicle size, particle size distribution, zeta potential, transmission electron microscopy (TEM), total drug content, small angle x-rays scattering (SAXS), headspace gas chromatography (HS-GC) for residual solvent estimation, in-vitro drug release. The optimized cubosomes of TAC had a vesicle size and PDI of 173.8 nm and 0.189 respectively with an entrapment efficiency of around 92.78 %. TEM analysis and SAXS of prepared sample confirmed a cubic shape of TAC loaded cubosomes. HS-GC of the optimized formulation suggested that the prepared formulation of TAC cubosomes have ethanol content of 64.96 ppm which was less than the permitted level of ethanol according to ICH guidelines Q3C (R6). Moreover, in-vitro drug release data of TAC cubosomes suggested a better release of drug from prepared cubosomes i.e. around 76.09 % in 24 hrs compared to the suspension of TAC in water i.e. 23.08 %.

11.1.5. FBX loaded cubosomes

To prepare the cubosomes of FBX bottom up approach was used. The cubosomes of FBX was optimized using QbD approach. During optimization of FBX loaded cubosomes, 2-level fractional factorial design was employed to screen out various Critical Quality Attributes (CQAs) which had significant impact on cubosomes of FBX. The CQAs which were screened out were particle size, particle size distribution and % entrapment efficiency. These CQAs were further optimized using 3^2 factorial design generated by Design Expert 7.0. An optimized batch was prepared using composition of

Critical Material Attributes-CMAs (GMO and PVA) suggested by overlay plot of 3^2 factorial design generated by Design Expert 7.0. Various characterization test of this optimized batch was performed like % entrapment efficiency, particle size, particle size distribution, zeta potential, transmission electron microscopy (TEM), total drug content, small angle x-rays scattering (SAXS), headspace gas chromatography (HS-GC) for residual solvent estimation, in-vitro drug release. The optimized cubosomes of FBX had a particle size and PDI of 157.5 nm and 0.165 respectively with an entrapment efficiency of around 85.2 %. TEM analysis and SAXS of prepared sample confirmed a cubic shape of FBX loaded cubosomes. HS-GC of the optimized formulation suggested that the prepared formulation of FBX cubosomes have ethanol content of 167.95 ppm which was less than the permitted level of ethanol according to ICH guidelines Q3C (R6). Moreover, in-vitro drug release data of FBX cubosomes suggested a better release of drug from prepared cubosomes i.e. around 61.08 % in 24 hrs compared to the suspension of FBX in water i.e. 32.07 %.

11.1.6. Microneedle (MN) patch loaded with optimized cubosomal formulation

MN patches were prepared using PVA-6000 (polyvinyl alcohol-6000) as matrix former and lactose as filler. MN patch loaded with cubosomes of TAC/FBX was optimized using 3^2 factorial design generated by Design Expert 7.0. In this design, axial fracture force of microneedle and its dissolution time were selected as CQAs. After preparing various batches generated by Design Expert 7.0, a data related to axial fracture force and dissolution time were fed to the Design Expert 7.0 and optimized area was generated which gave the proposed composition for the optimized batch. An optimized batch was prepared according to this and then, it was characterized for various parameters like axial fracture force, dissolution time, scanning electron microscopy, skin penetrability, pore closure kinetic, physical stability of cubosomes after its dissolution in water, in-vitro drug release, total drug content etc. Axial fracture force of MN Patch containing cubosomes of TAC and FBX were found to be 1.16 N and 1.2 N respectively which was sufficient to breach the stratum corneum. *In-vitro* dissolution time of prepared MN Patches containing cubosomes of TAC and FBX was performed and found out to be 1.50 and 1.25 min respectively. Images of SEM showed smooth surfaced, conical

microneedles having a length of 1.5 mm, and a base diameter of approx. 200 µm. Skin penetrability study proved that developed MN patch was able to penetrate the skin and deliver a drug to the dermis layer. According to pore closure kinetic study, it can be concluded that pores created by the MN patch was completely closed in 24 hrs. From data of in-vitro drug release study, it can be concluded that, in 2 hrs more than 90 % of drug was released from MN patch.

11.1.7. Ex-vivo characterization

For ex-vivo characterization of developed formulation, ex-vivo skin permeation and skin deposition, ex-vivo fluorescence microscopy to understand permeation behavior of developed formulation, and in-vitro cell viability study to established cell cytotoxicity of formulations. On the basis of the results of ex-vivo permeability from various formulations, they are organized in the order of increasing permeability: TAC Suspension < TAC MN Patch < TAC cubosomes < cubosomes of TAC loaded MNP. Similar order of drug permeation was also observed for the formulation developed for FBX. Further, an ex-vivo fluorescence microscopy of optimized formulation also supported the resulted obtained through ex-vivo permeation study. Form the data of cell cytotoxicity study, it was understood that cubosomes of TAC/FBX were less cytotoxic than suspension of TAC/FBX. The developed formulations of TAC/FBX were also characterized for histopathological evaluation which indicates that prepared formulations have no toxic effects on rat abdominal skin in compare to the skin which treated with isopropyl alcohol.

11.1.8. In-vivo study

In-vivo study was performed in stages: Pharmacokinetic study and pharmacodynamic study.

Pharmacokinetic study of developed formulation was performed on Wistar Rats. Various pharmacokinetic parameters like C_{max}, T_{max}, AUC, $T_{1/2}$, and MRT were found out during this study for various formulations of TAC/FBX. For MN patch of TAC, C_{max}, T_{max}, AUC, $T_{1/2}$, and MRT were found 3.80 ng/ml, 6.00 min, 120.81 ng*h/ml, 25.43 min and 37.92 hrs respectively which much better than data obtained for any other developed formulation of TAC. The relative bioavailability of TAC of cubosomes of TAC and MN patch of TAC loaded cubosomes were found to be 1.92 and 3.48 respectively in

comparison to the marketed oral capsule. Similarly, C_{max}, T_{max}, AUC, $T_{1/2}$, and MRT were also obtained for FBX which are 271. 03 ng/min, 6.00 min, 7328.7 ng*h/ml, 18.57 min and 29.05 hrs. A relative bioavailability of FBX of cubosomes of FBX and MN patch of FBX loaded cubosomes were found to be 1.37 and 2.63 respectively in compare to the marketed oral capsule.

Pharmacodynamic studies for both the drugs were also carried out and various parameters were observed during this study like paw volume and body weight. At the end of the study, X-ray of paw of rat was also taken in both studies. In case of TAC, level of Rheumatoid Factor was also recorded and in case of FBX level of uric acid was measured during study. From pharmacodynamic study it can be said that the prepared formulations were effective for the treatment of rheumatoid arthritis and gout.

11.1.9. Stability study

Stability study of prepared formulations was conducted at 40 ± 2 °C and 75 ± 5% RH and at the end of 1, 2 and 3 month the prepared formulations were tested for axial fracture force, in-vitro dissolution time (for MN patch), vesicle size, PDI and % entrapment for cubosomes of TAC/FBX. On storage, AFF was slightly decreased in all four formulations while there is no effect on in vitro dissolution time of developed MNP. Similarly, a slight increase in vesicle size and PDI as well as a slight decrease in drug entrapment was evident on storage in both cubosomal formulations. Thus, the MN patch was stored in air tight container with silica bag to prevent from losing its strength.

10.2. Conclusion

Arthritis is main cause of disability in humankind in developed and developing countries. Among which rheumatoid arthritis and gouty arthritis are most prevalent ones. Immunosuppressants and xanthine oxidase inhibitors are the choice of treatment for RA and gouty arthritis respectively. Thus, transdermal drug delivery systems of TAC (immunosuppressant) and FBX (xanthine oxidase inhibitors) were developed as their treatment options.

TAC is immunosuppressant which acts by inhibiting immune response of human body. Cubosomes of TAC were developed to improve the bioavailability of TAC. Then, these developed cubosomes were loaded into the microneedle patch to achieve better

permeation of drug through skin. The developed cubosomes of TAC were characterized for various parameters as described in chapter 5 and summarized in section 10.1.4. After loading it in MN patch it was also characterized for further evaluation parameters as described in chapter 7 and summarized in 10.1.6. Both optimized formulations were evaluated for ex-vivo characterization studies, pharmacokinetic and pharmacodynamic study which indicates its safety with better permeation across the skin and bioavailability compare to marketed formulations as described in chapter 8 and 9 and summarized in 10.1.7 and 10.1.8. According to the stability study of prepared formulations, it can be concluded that the prepared cubosomes of TAC were stable at room temperature while MN patch of TAC required special type of packaging which prevent moisture absorption by MN patch.

FBX is xanthine oxidase inhibitor which acts by inhibiting xanthine oxidase which is found in blood. Cubosomes of FBX were developed to improve the bioavailability of FBX. Then, these developed cubosomes were loaded into the microneedle patch so that better permeation of drug through the skin was achieved which has positive impact on bioavailability of FBX. The developed cubosomes of FBX were characterized for various parameters as described in chapter 6 and summarized in 10.1.5. After loading it in MN patch, it was also characterized for further evaluation parameters as described in chapter 7 and summarized in 10.1.6. Both optimized formulations were evaluated for ex-vivo characterization studies, pharmacokinetic and pharmacodynamic study which indicates its safety with better permeation across the skin and bioavailability compared to marketed formulations as described in chapter 8 and 9 and summarized in 10.1.7 and 10.1.8. According to the stability study of prepared formulations, it can be concluded that the prepared cubosomes of FBX were stable at room temperature while MN patch of FBX required special type of packaging which prevent moisture absorption by MN patch.