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PII: S0928-0987(17)30142-2
DOI: doi: 10.1016/j.ejps.2017.03.009
Reference: PHASCI 3949

To appear in: *European Journal of Pharmaceutical Sciences*

Received date: 21 December 2016
Revised date: 15 February 2017
Accepted date: 7 March 2017

Please cite this article as: Aswathi R Hegde, Prarthana V Rewatkar, Jyothsna Manikkath, Karnaker Tupally, Harendra S Parekh, Srinivas Mutalik , Peptide dendrimer-conjugates of ketoprofen: Synthesis and ex vivo and in vivo evaluations of passive diffusion, sonophoresis and iontophoresis for skin delivery. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Phasci(2017), doi: 10.1016/j.ejps.2017.03.009

Peptide dendrimer-conjugates of ketoprofen: Synthesis and *ex vivo* and *in vivo* evaluations of passive diffusion, sonophoresis and iontophoresis for skin delivery

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ABSTRACT

The aim of this study was to evaluate skin delivery of ketoprofen when covalently tethered to mildly cationic (2^+ or 4^+) peptide dendrimers prepared wholly by solid phase peptide synthesis. The amino acids glycine, arginine and lysine formed the dendrimer with ketoprofen tethered either to the lysine side-arm (N_ε) or periphery of dendrimeric branches. Passive diffusion, sonophoresis- and iontophoresis-assisted permeation of each peptide dendrimer-drug conjugate (D1-D4) was studied across mouse skin, both *in vitro* and *in vivo*. In addition, skin toxicity of dendrimeric conjugates when trialed with iontophoresis or sonophoresis was also evaluated. All dendrimeric conjugates improved aqueous solubility at least 5-fold, compared to ketoprofen alone, while also exhibiting appreciable lipophilicity. *In vitro* passive diffusion studies revealed that ketoprofen in its native form was delivered to a greater extent, compared with a dendrimer-conjugated form at the end of 24 h (Q_{24h} (μg/cm^2): ketoprofen (68.06±3.62) > D2 (49.62±2.92) > D4 (19.20±0.89) > D1 (6.45±0.40) > D3 (2.21±0.19). However, sonophoresis substantially increased the skin permeation of ketoprofen-dendrimer conjugates in 30 min (Q_{30min} (μg/cm²): D4 (122.19±7.14) > D2 (66.74±3.86) > D1 (52.10±3.22) > D3 (41.66±3.22) although ketoprofen alone again proved superior (Q_{30min}: 167.99±9.11 μg/cm²). Next, application of iontophoresis was trialed and shown to considerably increase permeation of dendrimeric ketoprofen in 6 h (Q_{6h} (μg/cm^2): D2 (711.49±39.14) > D4 (341.23±16.43) > D3 (89.50±4.99) > D1 (50.91±2.98), with a Q_{6h} value of 96.60±5.12 μg/cm^2 for ketoprofen alone). *In vivo* studies indicated that therapeutically relevant concentrations of ketoprofen could be delivered transdermally when iontophoresis was paired with D2 (985.49±43.25 ng/ml). Further, histopathological analysis showed that the dendrimeric approach was a safe mode as ketoprofen alone. The present study successfully demonstrates that peptide dendrimer conjugates of

ketoprofen, when combined with non-invasive modalities, such as iontophoresis can enhance skin permeation with clinically relevant concentrations achieved transdermally.

Keywords: Ketoprofen, dendrimeric conjugates, passive diffusion, sonophoresis, iontophoresis, skin permeation

1. INTRODUCTION

Historically, the skin was thought to be totally impervious to exogenous chemicals (Scheuplein and Blank, 1971). However, once it was implicit that the skin was a semi-permeable membrane rather than a totally impervious barrier; there arose new possibilities for the use of this route as a portal for systemic drug absorption. In general, the epidermis (more specifically the stratum corneum – the top-most skin layer) limits the delivery of drug molecules. Only low molecular weight drugs (generally <500 Da) with adequate physicochemical properties can be passively transported through skin (Prausnitz et al., 2004). Various approaches have evolved to expand the number of drugs delivered through skin using formulation manipulation like supersaturated solution, microemulsion, liposomal systems, and chemical enhancement using permeation enhancers such as polyalcohols, pyrrolidones, amines, amides, fatty acids, sulphoxides, esters, terpenes, alkanes, surfactants and phospholipids (Barry et al., 2004). Physical penetration enhancement methods like iontophoresis, sonophoresis, electroporation and magnetophoresis have also been studied with promising outcomes achieved (Patel and Trivedi, 2011).

In recent years, dendrimers have been identified as permeation enhancers for skin delivery of drugs. The development of dendrimers as potential drug vehicles or scaffolds now is one of the most active areas of biomedical and pharmaceutical sciences (Cheng and Xu, 2008). Dendrimers are hyperbranched macromolecules having a tree-like structure, consisting of a core from which alternating layers of monomers extend. They possess several features such as regular branching, multivalency, small-size, high density of surface-functional groups, extremely low polydispersity, host-guest entrapment properties and precise molecular weight. There are reports on the use of dendrimers, specifically poly(amidoamine) (PAMAM) systems, in transdermal

drug delivery prepared as PAMAM-drug conjugates (Najlah et al., 2006; Kumar et al., 2010). Although PAMAM dendrimers exhibited promise in the delivery of drugs like 5-fluorouracil, tamsulosin, indomethacin, ketoprofen, riboflavin and diflunisal, their biodegradation and inherent cytotoxicity remains an issue (Duncan & Izzo, 2005; Venuganti et al., 2008).

Peptide dendrimers have a wedge-like macromolecular structure. They are composed of amino acids connected via peptide/ amide bonds which are present within the branching core as well as on their outer surface. They have many advantages including lower toxicity, biodegradation as well as cost effectiveness to prepare in bulk. Encapsulation and conjugation of drugs with peptide dendrimers have been studied for delivery of hydrophobic drugs (Gajbhiye et al., 2008), and this study explores the application of peptide dendrimers for drug delivery, and is an extension of our earlier work (Mutalik et al., 2009, Mutalik et al., 2011; Mutalik et al., 2013; Mutalik et al., 2014; Shetty et al., 2017). In this study, we have synthesized dendrimeric conjugates of ketoprofen using different natural amino acids. The release of drug from a conjugate occurs via chemical or enzymatic cleavage of a hydrolytically labile bond.

Ketoprofen was chosen as a model drug in this study given is available in topical form, serving as a non-steroidal anti-inflammatory agent (NSAID) with analgesic and antipyretic properties. The importance of ketoprofen in the therapeutic field has stimulated the development of topical dosage forms to improve its percutaneous absorption (Moretti et al., 2000).

Pertaining to application of peptide dendrimers in skin/ transdermal delivery of bioactive molecules, we have previously studied the passive diffusion and the effect of sonophoresis and iontophoresis on the penetration of peptide dendrimers across human skin (Mutalik et al., 2012; Mutalik et al., 2013). Additionally, enhancement in deposition and permeation of 5-fluorouracil across human epidermis assisted by peptide dendrimers was also investigated (Mutalik et al.,

2014). There are few reports available on the conjugation of drugs such as paclitaxel and methotrexate with PAMAM dendrimers. However these reports revealed the possible risks associated with these PAMAM dendrimeric conjugates in therapeutic use (Myc et al., 2008; Cline et al., 2013; Satsangi et al., 2014). Having understood this, we have now transitioned to assess chemically conjugated ketoprofen-peptide dendrimers for transdermal drug delivery on which no reports are available in the literature. In addition, the effect of sonophoresis and iontophoresis on the permeation of these dendrimeric conjugates, along with histological evaluation was studied.

2. MATERIALS AND METHODS

2.1. Materials

Ketoprofen was kindly gifted by T & T Pharma Care Pvt. Ltd., Mumbai, India. Fmoc-Gly-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-Arg(Pbf)-OH, *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) and Rink amide resin were procured from Merck Biosciences AG, Darmstadt, Germany. Dichloromethane (DCM), acetonitrile and *N*,*N*-dimethylformamide (DMF) were purchased from RCI Labscan, Samutsakorn, Thailand. Trifluoroacetic acid (TFA), *N*,*N*-diisopropylethylamine (DIEA), triisopropylsilane (TIPS), *N*-methyl pyrrolidine (NMP), HEPES, piperidine, diethyl ether, and acetaminophen were purchased from Sigma-Aldrich, St. Louis, Mo., USA. Heptafluorobutyric acid (HFBA) was obtained from Fluka Chemie GmbH, Buchs, Switzerland.

Male Swiss Albino mice (6-8 weeks old; 20-25 g) were used in this study. The animal experimental protocol was approved by Institutional Animal Ethical Committee, Kasturba Medical College, Manipal University, Manipal (Approval No.: IAEC/KMC/12/2014).

2.2. Synthesis, purification and characterization of dendrimeric conjugates of ketoprofen

In total, four peptide dendrimeric conjugates of ketoprofen, having either 2^+ or 4^+ charge were synthesized by Fmoc Solid Phase Peptide Synthesis (SPPS) (Mutalik et al., 2011). Details of the dendrimeric conjugates are given in Table 1 and the synthesis scheme is given in Figure 1. Rink-amide resin was treated with DMF and Fmoc removal achieved with 20% v/v piperidine in DMF. Firstly, Fmoc-Gly-OH activated with HBTU and DIEA was coupled to the resin. The resin was then washed with DMF, and treated with 20% v/v piperidine in DMF prior to coupling of the next amino acid. This successive process was continued until the required dendrimeric conjugates of ketoprofen were formed. At each step of amino acid coupling, the coupling efficiency was established by the Ninhydrin test. Next amino acid was coupled only after achieving at least 99% coupling of the preceding amino acid. Once the desired peptide dendrimeric conjugate of ketoprofen was synthesized, terminal Fmoc groups were detached. This step was followed by flow washing with DMF, then DCM and vacuum-drying of the resin. The peptide dendrimer-ketoprofen conjugates were then separated off-resin by stirring the product in a mixture of TFA, DCM, water and TIPS (90:5:2.5:2.5) for 3-4 h. The resin mixture was then filtered, and TFA eluent removed *in vacuo*. The subsequent residue was treated with toluene (3 x 50 mL), mixed vigorously with ice-cold diethyl ether (3 x 20 mL) and lyophilized.

The dendrimeric conjugates of ketoprofen were purified using a preparative HPLC (Waters, Milford, MA, USA) and subjected to mass spectrometry (MS) analysis, differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR) and zeta potential measurements. In MS analysis, all the synthesized dendrimeric conjugates of ketoprofen (D1,

D2, D3 and D4) were analyzed using ESI^+-MS (2000 QTRAP Nano spray™, MDS Sciex, a division of MDS Inc., Ont., Canada) for their molecular ion $[M+H]^+$.

For DSC analysis, the sample (plain ketoprofen and D1, D2, D3 or D4 conjugates) was sealed in an aluminium pan and scanned using a Differential scanning calorimeter (DSC-60; Shimadzu, Kyoto, Japan) between 25 and 300 °C, under nitrogen flow (30 mL/ min), at a heating rate of 5 °C/min. The reference used was an empty aluminium pan. Temperature calibration was done using indium as the standard (Devarakonda et al., 2005).

Infrared spectroscopy was performed using IR Spectrophotometer (FTIR 8300 Spectrophotometer, Shimadzu, Kyoto, Japan). The samples were mixed with KBr (200-400 mg) and compressed into discs by applying a pressure in a hydraulic press. The pellet was placed in the light path and the spectrum was recorded in the region of 4000 to 400 cm^{-1}.

Zeta potential was measured using a Zetasizer (Nano ZS, Malvern Instruments, UK). Ketoprofen, D1, D2, D3 or D4 were prepared in HEPES buffer pH 7.4 to get a concentration of 1 mg/ml each. Ten measurements were taken for each sample and the mean was reported.

2.3. Solubility Studies

Ketoprofen alone or dendrimeric conjugates (D1, D2, D3 and D4) were added to Ria tubes containing 3 mL of water. Excess quantity was added to ensure that the drug was saturated. The vials were kept on Rotospin rotary mixer (Tarsons, Kolkata, India) at 30 rpm for 24 h. The samples were passed through 0.45 µm membrane filter and diluted appropriately with water. The amount of drug dissolved was determined by using HPLC.

2.4. Partition coefficient (PC) determination

Saturated solutions of plain ketoprofen, D1, D2, D3 and D4 in Milli-Q water were prepared and the initial concentration of drug in the aqueous phase was analyzed by HPLC. The

volume of n-octanol used for the determination of partition coefficient was 1 mL and the final n-octanol/water phase ratio was 1:1. Known volumes of saturated solution of ketoprofen/ dendrimeric conjugates were mixed with same volume of n-octanol and the mixture was subjected to shaking using Rotospin rotary mixer (Tarsons, Kolkata) for 24 h. The partition coefficient was calculated using following equation:

$$\text{Partition coefficient} = (\text{drug})_{oil}/(\text{drug})_{aqueous}$$

2.5. Cleavage of ketoprofen from its dendrimeric conjugate

Trypsin was used to demonstrate cleavage of ketoprofen from its dendrimeric conjugates. All the conjugates were mixed with trypsin (1:3 molar ratios) and kept under stirring for 4 h at 37 ºC. At the end of four hours, samples were withdrawn and subjected to MS analysis to check for the presence of drug peak.

2.6. *In vitro* skin permeation studies (passive diffusion)

The diffusion cells (vertical type) with a diffusion area of 1.13 cm^2 and receptor compartment volume of 3.5 ml were used in the *in vitro* skin permeation studies. The abdominal portion of the male Swiss Albino mice was shaved with an electrical clipper on the previous day of the experiment. After sacrificial, the abdominal skin of the mice was excised, subcutaneous fat was removed and the skin was washed under running water. Then the skin was fixed in the diffusion cell and the stratum corneum faced the donor compartment. Donor solution comprised of 15 mM HEPES +75 mM NaCl and its pH adjusted to 7.4 with 1 M NaOH. Receptor solution comprised of 20 mM HEPES + 50 mM NaCl and its pH adjusted to 7.4 with 1 M NaOH to mimic the *in vivo* ionic concentration (Mutalik et al., 2013). This pH was selected taking into consideration the possibility of irritation or damage to the skin at lower and higher pH values (Jalwal et al., 2010). The whole cell system was maintained on a magnetic stirrer and the

receptor solution was stirred using a magnetic bead at a speed of 600 rpm. Drug dispersion (5 mg/mL in HEPES donor solution, pH 7.4) was added to the donor compartment and HEPES receptor buffer pH 7.4 was added to the receptor compartment. Similarly saturated solutions of D1, D2, D3 and D4 in HEPES donor solution (pH 7.4) were used in donor compartment. Samples (0.5 mL) were collected from the receptor compartment at time different time intervals between 0 and 24 h and an equal volume of plain receptor solution was replaced each time into the receptor compartment. The mixture was filtered through 0.45 µ and the amount of ketoprofen permeated was assayed by HPLC.

2.7. *In vitro* skin permeation studies with application of ultrasound (sonophoresis)

These experiments were conducted in a manner similar to the above, with ultrasound applied to the donor compartment. Ketoprofen dispersion (5 mg/mL) or its dendrimeric conjugates (D1, D2, D3 and D4) in pH 7.4 HEPES solution were added to the donor compartment and low frequency ultrasound was applied using a probe sonicator (VibraCell VC 130, Sonics and Materials, Newton, CT, USA). The tip of the probe was maintained at a distance of 3-5 mm from the skin. Ultrasound was applied for 30 min at the amplitude of ~30 to attain a power output of 7-8 W/cm^2 (Mutalik et al., 2011). Sample collection was performed at the end of 15 and 30 min. On-off cycle of 1 sec was followed, i.e. ultrasound on for 1 sec, then off for 1 sec, again on for 1 sec, then off for 1 sec. This was followed to prevent drastic increase in temperature in during sonication. Replacement of donor solution every 2 min was also done to prevent the increase in temperature. For example: collection of 15th minute sample involved 2 min sonophoresis; 1 min- donor solution replacement; 2 min- sonophoresis; 1 min- donor solution replacement; 2 min- sonophoresis; 1 min- donor solution replacement; 2 min sonophoresis and 1 min donor solution replacement. Samples of 0.5 mL were collected from the

receptor compartment and an equal volume of buffer was replaced. The mixture was filtered through 0.45 µ and analyzed by HPLC.

2.8. *In vitro* skin permeation studies with application of electric current (iontophoresis)

Horizontal diffusion cells of 2.5 mL capacity were used for all *in vitro* iontophoretic studies. Silver (Ag) rod and silver chloride (AgCl) rod were used as anode and cathode, respectively. Silver-Silver chloride (Ag-AgCl) electrodes were used as they do not cause electrolysis of water and hence would not lead to pH shifts in the solution (Rootare et al., 1977). A constant current (D.C.) of 0.38 mA/cm^2 was applied in all the experiments. The dispersion of ketoprofen or D1, D2, D3 and D4 conjugates in HEPES solution of pH 7.4 were added to donor compartment. Samples (0.4 mL) were withdrawn at different intervals between 0 and 6 h from the receptor compartment. The mixture was filtered through a 0.45 µm membrane and analyzed by HPLC.

2.9. *In vivo* skin permeation study of ketoprofen and dendrimeric conjugate (D4) – application of ultrasound

The animals were divided into 2 groups of 6 animals each. One group was treated with plain ketoprofen and the other one was treated with D4 conjugate. Male mice (whose abdominal fur had been shaved off on the previous day) were anaesthetized by i.p. administration of ketamine hydrochloride. After about 10 min into anesthesia, the mice were fixed on their backs and the donor compartment of the vertical diffusion cell was glued on the mice abdomen using minimal amount of grease on the outer edge of the flange. Adhesive tape was also used to prevent leakage of the drug solution from the diffusion cell. The donor compartment was filled with ketoprofen or D4 dendrimeric conjugate dispersion (5 mg/mL) in HEPES solution of pH

7.4. Ultrasound was applied by immersing the transducer in the donor solution. The location of the center of the diffusion cell was chosen so as to prevent application of ultrasound directly to bone close to the surface, which might have caused damage to the blood capillaries around the bone. The tip of the probe was maintained at a distance of 3-5 mm from the skin. The ultrasound application procedure was similar to the *in vitro* experiments. At the end of 15 min and 30 min, blood (about 300 µL) was withdrawn by retro orbital puncture into heparinized tubes. The blood samples were immediately centrifuged to separate the plasma and the latter was stored at -20°C. The amount of ketoprofen in the samples was estimated by HPLC.

The abdominal skin was excised and subjected to histopathological studies after H & E staining microscopically with respect to different parameters such as degeneration, necrosis, congestion, inflammation, cavitation and edema. The histopathological parameters were scored using the following scale:

Histopathological scale: + = slight; ++ = moderate; +++ = severe.

2.10. *In vivo* skin permeation study of ketoprofen or dendrimeric conjugate (D2) – application of electric current

This was carried out in a manner similar to the above procedure. The donor compartment was filled with ketoprofen or D2 dendrimeric conjugate dispersion (5 mg/mL) in HEPES solution of pH 7.4. One of the electrodes was placed in the donor solution without touching the skin. The tip of other electrode was wound with cotton dipped in receptor solution and placed at an area close to the breast to complete the electrical circuit. The animals were divided into 2 groups of 6 animals each. One group was treated with ketoprofen (cathodic iontophoresis) whereas the other was treated with D2 (anodic iontophoresis). At the end of 1 and 3 h, blood (about 300 µL) was withdrawn by retro orbital puncture into heparinized tubes. The blood

samples were immediately centrifuged to separate the plasma and the latter was stored at -20 °C. The drug content in the samples was determined by HPLC. The abdominal skin was excised and subjected to histopathological studies microscopically after H&E staining as explained above.

2.11. HPLC analysis of ketoprofen

Analysis was done by modifying a previously reported RP-HPLC method (Ahmed and Fatahalla, 2007; Boppana et al., 2015; Manikkath et al., 2017). Shimadzu (Kyoto, Japan) HPLC system consisted of a degasser (DGU 20As Prominence), a system controller (CBM-20A Prominence), pumps (LC-10 AD), an auto-injector (SIL-10AXL) and a variable-wavelength UV/Vis detector (SPD-10A). The chromatographic data was analyzed using LC solution 1.24 SP1 software. The HPLC column was a reverse phase C18 column (RP-C18 Genesis; particle size 5 µm; 250 × 4.6 mm), maintained at 25 ºC. Acetonitrile and potassium dihydrogen phosphate buffer (pH 3, 10 mM) mixture (55:45) was used as the mobile phase at a flow rate of 0.8 mL/min. The total run time for each sample was 20 min. The detector wavelength was set at 260 nm and the volume of injection volume was 100 µL. The retention time of ketoprofen was found to be 8.06 min and there were no interfering peaks from skin. The bio-analytical method was developed in a similar manner (Boppana et al., 2015). Lercanidipine was used as the internal standard (IS). The retention time of ketoprofen was found to be 7.6 min and that of the IS was 10.3 min.

3. RESULTS AND DISCUSSION

3.1. Dendrimer Synthesis and Purification

Dendrimeric conjugates of ketoprofen containing varying positive charges were synthesized by Fmoc SPPS (Figure 1). The chemical structures of the peptide dendrimeric

conjugates of ketoprofen are shown in Figure 2. The yield of all dendrimers after purification was >70%. In their characterization using ESI^+-MS (QTRAP LC/MS/MS system), all dendrimers showed the desired molecular ion $[M+H]^+$, confirming the formation of respective peptide dendrimeric conjugates (Table 1).

3.2. Chemical and physicochemical Characterization of conjugates

3.2.1. Mass spectrometry results:

From the MS spectral analysis it was observed that the dendrimeric conjugates showed the desired molecular ion $[M+H]^+$, *viz.*, D1, D2, D3 and D4 showed the respective $[M+H]^+$ peaks of 879.53, 823.52, 987.52 and 931.51, confirming the formation of respective conjugates. The $[M+H]^+$ peak corresponding to plain ketoprofen was absent in these MS spectra. The results are shown in Table 1 and Figure 3.

3.2.2. FTIR Analysis

The results of FTIR spectroscopy (Figure 4) were indicative of the formation of CONH bond and thereby support of the formation of peptide dendrimeric conjugates of ketoprofen. The IR spectrum of ketoprofen, as shown in Figure 4A, exhibited characteristic peaks at 3730 cm^{-1} (OH group), 3026.31 cm^{-1} (aromatic C-H stretch), 2993 cm^{-1} (C-H alkane stretch), 2937.59 cm^{-1} (CH_3 group), 2360.87 cm^{-1} (carboxylic acid group), 1697 cm^{-1} (COOH containing C=O group), 1654.36 cm^{-1} (C=O group), 1579.70 cm^{-1} (aromatic -C=C- bend).

FTIR spectra of D1 (Figure 4B) exhibited merged peaks at 3591.46 cm^{-1} (-NH-; 2º amine), 3070.68 cm^{-1} (=C-H stretch), 2939.52 cm^{-1} (C-H alkyl stretch), 1739 cm^{-1} (C=O stretch), 1678.07 cm^{-1} (C=O group) and 1653 cm^{-1} (C=N group, indicating the presence of amino acids).

FTIR spectra of D2, as shown in Figure 4C, exhibited peaks at 3059.10 cm^{-1} (=C-H stretch), 3670.54 cm^{-1} (O-H stretch), 2941.44 cm^{-1} (C-H alkyl stretch), 1678.07 cm^{-1} (C=O

group) and 1653 cm^{-1} (C=N group, indicating the presence of amino acids), 1454.33 cm^{-1} (C-H bend alkanes).

FTIR spectra of D3 exhibited peaks at 3564.45 cm^{-1} (O-H stretch), 2939.52 cm^{-1} (C-H alkyl stretch), 1739.79 cm^{-1} (C=O stretch), 1653 cm^{-1} (C=N group, indicating the presence of amino acids), 1541.12 cm^{-1} (C-C aromatic stretch), 1454.33 cm^{-1} (C-H bend alkanes), 1315.45 cm^{-1} (C-O stretch), 1047 cm^{-1} (C-N stretch, aliphatic amine) as shown in Figure 4D.

FTIR spectra of D4 (Figure 4E) exhibited peaks at 3610.74 cm-1 (O-H stretch), 3277.06 cm^{-1} (N-H stretch), 3061.03 cm^{-1} (C-H aromatic stretch), 2939.52 cm^{-1} (C-H alkane stretch), 1678.07 cm^{-1} (C=O stretch), 1516.05 cm^{-1} (C-C aromatic stretch), 1454.33 cm^{-1} (C-H bend alkanes), 1286.52 cm^{-1} (C-O stretch), 1201.65 cm^{-1} (C-N stretch).

3.2.3. Differential Scanning Calorimetry (DSC) Results

The DSC thermograms are given in Figure 5. The DSC thermogram of pure ketoprofen showed a sharp endothermic peak at 99.5 ºC, corresponding to its melting point. In all the thermograms of the dendrimeric conjugates of ketoprofen, the peak of ketoprofen was absent. Endothermic peaks with low intensity were observed in the thermograms of the dendrimeric conjugates; near the melting points of lysine, glycine and arginine (224, 245 and 235 ºC, respectively). In a previous report also, the drug peak disappeared when complexed with PAMAM dendrimer which was attributed to decreased heats of fusion of the drug in the presence of dendrimers (Devarakonda et al., 2005). These results are collectively in favor of formation of ketoprofen-dendrimer conjugates.

3.2.4. Zeta potential Results

Zeta potential of all the dendrimeric conjugates of ketoprofen exhibited positive charge at pH 7.4 (D1=0.91±0.02 mV; D2=6.95±0.12 mV; D3=1.61±0.05 mV; D4=6.17±0.09 mV).

Ketoprofen showed a zeta potential of -12.5±0.81 mV. The positive zeta potential values of the conjugates are clearly indicative of the conjugation of ketoprofen with peptide dendrimer (Mutalik et al., 2012).

3.2.5. Solubility studies

All the dendrimeric conjugates showed higher solubility in water as compared to ketoprofen (58.54±0.21 µg/mL). Among the conjugates, D4 showed the highest solubility, (558.31±15.1 µg/mL) followed by D2 (392.50±11.1 µg/mL), D1 (317.98±9.2 µg/mL) and D3 (267.02±5.6 µg/mL). This confirms the hydrophilic nature of dendrimeric conjugates of ketoprofen in comparison with plain drug. These results are in agreement with previous results where PAMAM dendrimers exhibited enhanced solubility for ketoprofen. The extent of increase in solubility of ketoprofen with peptide dendrimers was noted to be less than that attained with PAMAM dendrimers; however, the PAMAM dendrimers were of a higher generation (charge) and MW (Yiyun et al., 2008).

3.2.6. Partition coefficient studies

Ideal drug candidates for transdermal drug delivery should possess Log *P* between -1 to 4 (Panchagnula, 1997). Plain ketoprofen (Log *P* value: 1.22) and all the synthesized dendrimeric conjugates of ketoprofen also showed the Log *P* values between -1 to 4 indicating sufficient lipophilicity as required for skin delivery of the conjugates. The Log *P* value of ketoprofen obtained in this study is in close agreement with previous reports (Schmitt and Guentert, 1990). The lipophilicity of the conjugates was ordered as follows, with D2 conjugate having highest lipophilicity:

D2 (-0.24±0.02) > D4 (-0.74±0.05) > D1 (-0.81±0.06) > D3 (-0.99±0.07).

From the partition coefficient measurements there is an evidence to indicate that conjugation of peptide dendrimers with ketoprofen alters its solubility and partition coefficient, suggesting that it may therefore alter the skin partitioning.

3.3. Cleavage of ketoprofen from its dendrimeric conjugate

It is important that ketoprofen should be released/ cleaved off from its conjugated form when it enters the skin. Trypsin, present in the skin, selectively cleaves the molecules with amino acids such as lysine and arginine. Koshikawa et al. (1998) demonstrated that trypsin is extensively expressed in the cells lining the skin, stomach, small intestine, liver and kidney as well as in the leukocytes of the spleen and nerve cells in the brain. Since all our synthesized peptide dendrimeric conjugates contain these amino acids, trypsin was used to observe whether ketoprofen would be cleaved/ released within the skin from its conjugated form. As discussed previously in Section 3.2.1, MS spectra of dendrimeric conjugates did not show the presence of free ketoprofen. It is expected that if the cleavage takes place, then MS analysis should reveal the molecular ion of ketoprofen. The results indicated the presence of $[M+H]^+$ peak of ketoprofen (255.40) in all MS spectra obtained with D1 to D4 peptide dendrimeric conjugates after treating with trypsin. These observations clearly demonstrate the cleavage/ release of ketoprofen from its conjugated form within the skin. The typical MS chromatogram of cleavage study with D1 conjugate is shown in Figure 6.

3.4. *In vitro* skin permeation studies

3.4.1. *In vitro* passive diffusion studies

The results of permeation of plain ketoprofen and its dendrimeric conjugates across mouse skin are shown in Figure 7. The amount of ketoprofen permeated at the end of 24 h (Q_{24h})

was 68.06±3.62 µg/cm^2. These results are supported by the data of previous studies where ketoprofen permeation was about 75 µg at the end of 24 h across the rat skin (Yiyun et al., 2007). It was observed that permeation of ketoprofen from its conjugates was considerably less when compared to plain ketoprofen. This could be due to high molecular weight (MW ≈1000) of the dendrimeric conjugates. Among the conjugates the permeation rate followed the pattern as shown below:

Q_{24h} (µg/cm^2): D2 (49.62±2.92) > D4 (19.20±0.89) > D1 (6.45±0.40) > D3 (2.21±0.19).

These Q_{24h} values are in accordance with partition coefficient results (Section 3.2.6). Lipophilicity of the penetrant is an important parameter that determines the extent of skin permeation. To a certain extent, higher lipophilicity of the molecules results in higher skin permeation. These results also indicate the importance of charge and MW of the conjugates. Higher permeation was observed with D2 conjugate (higher charge of 4^+ and low MW). Similarly less permeation was observed with D3 conjugate (lower charge of 2^+ and higher MW). However D1 and D4 did not exhibit expected dependency on MW and charge. This suggests that a combination of multiple factors such as partition coefficient, architecture, surface charge and MW influence permeation.

3.4.2. *In vitro* skin permeation studies with application of ultrasound (sonophoresis)

Once the passive diffusion of ketoprofen and its dendrimeric conjugates was studied, next step was to evaluate the effect of ultrasound on the transdermal permeation of ketoprofen in its plain- as well as in conjugated form. Sonophoresis is an effective strategy over passive diffusion in the transdermal and topical delivery (Herwadkar et al., 2012). The results of sonophoretic transdermal permeation of ketoprofen and its dendrimeric conjugates for 30 min are shown in Figure 8.

Appreciable amount of ketoprofen was permeated in 30 min with the aid of ultrasound. The amount of ketoprofen permeated from its plain form as well as from its dendrimeric conjugates in 30 min (Q_{30min}) with sonophoresis was more than the amount that permeated in 24 h with simple passive diffusion study. Highest skin permeation was shown by plain ketoprofen (Q_{30min} = 167.99±9.11 µg/cm^2) upon ultrasound application when compared to conjugates. This again could be attributed to the low molecular weight and high partition coefficient value of plain ketoprofen. The permeation rate of conjugates followed the pattern as shown below:

$Q_{30\ min}$ (µg/cm^2): D4 (122.19±7.14) > D2 (66.74±3.86) > D1 (52.10±3.22) > D3 (41.66±3.22).

The skin permeation trend observed with ultrasound application did not completely rely on MW or lipophilicity of dendrimeric conjugates.

For example: D4 conjugate ($[M+H]^+$=931.51), despite having higher MW than D1 ($[M+H]^+$=879.53) and D2 ($[M+H]^+$=823.52), exhibited higher permeation upon application of ultrasound. Likewise, D2 (Log *P*= -0.24) with high lipophilicity exhibited lower Q_{30min} value in comparison with D4 (Log *P*= -0.74) which showed highest skin permeation upon ultrasound application although its partition coefficient value was less than that of D2. Hence the trend observed in the skin permeation of dendrimeric conjugates in sonophoresis did not follow MW as well as partition coefficient dependency completely. There may be some other factors that play important role in transdermal permeation of these peptide dendrimeric conjugates of ketoprofen.

3.4.3. *In vitro* skin permeation studies with application of electric current (iontophoresis)

Iontophoresis improves the delivery of polar molecules as well as high molecular weight compounds such as peptides and oligonucleotides (Escobar-Chávez et al., 2012). In a previous

study, iontophoresis increased the permeation of ketoprofen through human cadaver skin in comparison to passive diffusion (Panus et al., 1997).The results of iontophoresis of ketoprofen and its dendrimeric conjugates are shown in Figure 9. The amount of ketoprofen permeated at the end of 6 h (Q_{6h}) observed with cathodic iontophoresis of ketoprofen was found to be 96.60±5.12 µg/cm^2.The rate of permeation of the drug with the application of iontophoresis with different conjugates (anodic iontophoresis) was observed in the following sequence:

Q_{6h} (µg/cm^2): D2 (711.49±39.14) > D4 (341.23±16.43) > D3 (89.50±4.99) > D1 (50.91±2.98).

D2 conjugate (with low MW and high positive charge) showed highest skin permeation rate for ketoprofen with the assistance of electric current. However a similar trend was not wholly evident with other dendrimeric conjugates.

With respect to lipophilicity, D2 and D4 conjugates with comparatively higher partition coefficient values (and hence higher lipophilicity), showed greater skin permeation rates in iontophoresis. However same dependency on partition coefficient values was not observed for other two (D1 and D3) conjugates. D1 conjugate (Log *P*= -0.81) exhibited lower skin permeation than D3 conjugate (Log *P*= -0.99), despite being more lipophilic comparatively.

On the other hand, the Q_{6h} values obtained with iontophoresis were in agreement with the zeta potential values of the dendrimeric conjugates. The zeta potential values of dendrimeric conjugates were in the order of: D2 > D4 > D3 > D1 (Section 3.2.4) and a similar trend was observed with iontophoresis mediated skin permeation rate for different conjugates. However more detailed studies are required to confirm the mechanism of transport of these dendrimeric conjugates.

The interesting point to observe in this iontophoresis study is the lower skin permeation rate of plain ketoprofen (upon cathodic iontophoresis) in comparison with D2 and D4, although ketoprofen possesses comparatively lower molecular weight and higher Log *P* values. This may be attributed to the perm-selectivity of the skin. The isoelectric point for mouse skin was found to be slightly below 3.8 (Alvarez et al., 1998). The skin is positively charged at pH below the pI and shows anion perm-selectivity. When pH is above pI, the skin is negatively charged and hence illustrates cation perm-selectivity. The net positive charge of the conjugates and the pI dependent cation perm-selectivity of the skin would be responsible for greater permeation of ketoprofen from conjugates upon anodic iontophoresis in comparison with cathodic iontophoresis of plain ketoprofen.

3.4.4. Comparison of permeation strategies

When different permeation strategies employed in this study *viz*. passive diffusion, sonophoresis and iontophoresis were compared each other for plain ketoprofen, it was observed that permeation of drug was highest with sonophoresis (Q_{30min}: 167.99±9.11 µg/cm^2) followed by iontophoresis (Q_{6h}: 96.60±5.12 µg/cm^2) and least with passive diffusion (Q_{24h}: 68.06±3.62 µg/cm^2).

For the dendrimeric conjugates, iontophoresis was found to be appropriate mode to ensure highest permeation of drug. Anodic iontophoresis of D2 conjugate showed highest permeation (Q_{6h}: 711.49±39.14 µg/cm^2), which was over 7-fold greater than that achieved with ketoprofen alone. On the other hand, sonophoretic delivery of ketoprofen from its dendrimeric conjugates was lower than that observed with sonophoretic delivery of plain drug, and a similar trend was also observed with passive diffusion.

Hence, the results of the present study suggest that chemical conjugation of ketoprofen to peptide dendrimers may not be a suitable approach to enhance the permeation of drug via passive diffusion or sonophoresis. The results demonstrate the need of free peptide dendrimer at the site to enhance the permeation of bioactive molecules. Our previous report (Mutalik et al., 2014) on the enhancement of transdermal permeation of 5-fluorouracil demonstrated that the presence of free peptide dendrimer, which acts as transdermal permeation enhancer, significantly increased the skin permeation of drug.

Chemical conjugation of ketoprofen to peptide dendrimer considerably enhanced the skin permeation of ketoprofen via iontophoresis. This might be due to the presence of positive charge on the dendrimer, which even after conjugation drives the molecule into the skin when an electric current is applied. In support of this, our previous studies have shown that iontophoresis can be successfully applied to enhance the transdermal permeation of peptide dendrimers (Mutalik et al., 2012). Electro-osmosis might also have played a more active role in transdermal permeation of higher MW compounds under the influence of an applied current (Guy et al., 2000; Mutalik et al., 2012). Overall, these results demonstrate that iontophoresis is a more favorable approach for permeation of dendrimeric conjugates of ketoprofen across the skin as compared to sonophoresis and passive diffusion.

Highest skin permeation among the dendrimeric conjugates was observed with D2 conjugate (iontophoretic delivery) and D4 conjugate (sonophoretic delivery) and hence this was selected for further permeation studies *in vivo*.

3.5. *In vivo studies*

3.5.1. *In vivo* skin permeation studies with the application of sonophoresis

In vivo skin permeation of ketoprofen and its dendrimeric conjugate (D4) in the presence of sonophoresis was studied in male Swiss Albino mice and the results are shown in Table 2. Plasma concentration of ketoprofen was higher with plain ketoprofen as compared to the dendrimeric conjugate (D4). These *in vivo* results are in support of our *in vitro* sonophoretic skin permeation results where plain ketoprofen showed better skin permeation than the conjugates, which can be attributed to the considerably lower MW of ketoprofen, compared to D4 conjugate.

3.5.2. *In vivo* skin permeation studies with the application of iontophoresis

Iontophoresis assisted *in vivo* skin permeation study of ketoprofen and D2 conjugate was studied. The results (Table 3) indicated that anodic iontophoresis of dendrimeric conjugate exhibited higher plasma concentration of ketoprofen as compared to cathodic iontophoresis of plain ketoprofen. The reasons as explained under Section 3.4.3 (*In vitro* skin permeation studies with application of electric current (iontophoresis)) may also be responsible for this greater *in vivo* skin permeation of ketoprofen from D2 conjugate under the influence of electric current. Our previous reports have demonstrated the usefulness of peptide dendrimeric structures in augmenting the permeation of 5-fluorouracil across human skin (Mutalik et al., 2009; Mutalik et al., 2013; Mutalik et al., 2014).

The *in vivo* skin permeation trend observed with sonophoresis and iontophoresis is in accordance with the results obtained with *in vitro* skin permeation of ketoprofen and its dendrimeric conjugates. From the results of *in vivo* permeation studies, it can be established that synthesizing ketoprofen-peptide dendrimeric conjugates is beneficial for iontophoresis mediated skin delivery of ketoprofen as compared to ultrasound-assisted delivery.

3.5.3. Absorption of ketoprofen: Transdermal route *vs*. oral administration

Male Swiss albino mice were orally administered with 20 mg/kg of ketoprofen. The plasma concentration of ketoprofen observed at the end 1 h after oral administration was compared with that observed with that observed with D2 conjugate + iontophoresis. The results indicated that the absorption of ketoprofen after oral administration was rapid and higher than the transdermal application of ketoprofen and its dendrimeric conjugate in combination with electric current. The plasma concentrations of ketoprofen after transdermal iontophoresis application and oral administration were 985.49±43.25 ng/ml (with 1 cm^2 of application area) and 8215.56±76.56 ng/ml, respectively. The results clearly demonstrate that the concentration of ketoprofen that is observed with oral route can easily be achieved with transdermal iontophoresis of peptide dendrimeric conjugate (D2) within an appreciable area of application. In this regard, peptide dendrimeric conjugates of ketoprofen can be se successfully applied to enhance the transdermal permeation of the drug iontophoretically and to deliver the drug at therapeutically effective concentration to systemic circulation through the skin in a non-invasive mode.

3.5.4. Histopathological evaluation of treated and untreated skin

In the histolopathological evaluations, normal skin did not show any indication of dermal toxicity. Mice treated with ketoprofen + sonophoresis as well as D4 + sonophoresis showed slight (scale: +) necrosis, inflammation and cavitation. Additionally D4 + sonophoresis showed slight (scale: +) degeneration. The skin treated with ketoprofen + iontophoresis exhibited slight (scale: +) inflammation and necrosis. Skin treated with D2 + iontophoresis showed slight (scale: +) inflammation, degeneration and necrosis. These results showed higher dermal safety and non-toxic nature of ketoprofen or dendrimeric conjugates in conjunction with iontophoresis/ sonophoresis.

4. CONCLUSIONS

Peptide dendrimeric conjugates of ketoprofen were synthesized using different amino acids by Fmoc SPPS. Next, MS, DSC, FTIR and zeta potential measurements confirmed the formation of dendrimeric conjugates of ketoprofen. All the peptide dendrimeric conjugates of ketoprofen showed higher aqueous solubility and appropriate lipophilicity. The conjugates also demonstrated cleavage/ release of ketoprofen when treated with trypsin. Ketoprofen-peptide dendrimeric conjugates benefit markedly from iontophoresis-mediated skin delivery as compared to ultrasound assisted delivery. *In vivo* results indicated that therapeutic concentrations of ketoprofen could be achieved iontophoretically with D2 conjugate within the area of skin application. Importantly, no toxicity was seen with either ketoprofen or dendrimeric conjugates in conjunction with sonophoresis or iontophoresis. The present study successfully demonstrates the application of chemical conjugates of drug and peptide dendrimers in enhancing the skin delivery of drugs.

ACKNOWLEDGEMENTS

Authors express their sincere thanks to T & T Pharma Care Pvt. Ltd., Mumbai, India for providing gift sample of ketoprofen. The authors are thankful to the Manipal College of Pharmaceutical Sciences, Manipal University, Manipal, India and School of Pharmacy (PACE), The University of Queensland, Brisbane, Australia for providing the necessary facilities.

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Table 1. Details of different peptide dendrimeric conjugates of ketoprofen synthesized

Type of dendrimeric conjugate	Dendrimer sequence (C-to-N terminus)	$[M+H]^+$ peak and MW (calculated)
D1 (Arginine 4^+)	Gly-Lys(Keto)-Lys-$(Arg)_2$	879.53; 878.52
D2 (Lysine 4^+)	Gly-Lys(Keto)-Lys-$(Lys)_2$	823.52; 822.51
D3 (Arginine 2^+)	Gly-Lys-$(Arg\text{-}Keto)_2$	987.52; 986.51
D4 (Lysine 2^+)	Gly-Lys-$(Lys\text{-}Keto)_2$	931.51; 930.50

Table 2. *In vivo* permeation of ketoprofen and its dendrimeric conjugate in the presence of sonophoresis

Treatment	Plasma concentration of ketoprofen (ng/ml)*	
	15 min	**30 min**
Ketoprofen + Sonophoresis	231.42 ± 9.88*	384.91 ± 16.22*
D4 + Sonophoresis	111.56 ± 6.48	134.59 ± 9.12

All the values are expressed as Mean±SD, n=6; * significantly different ($p<0.05$) compared to D4+sonophoresis at the respective time intervals.

Table 3. *In vivo* skin permeation of ketoprofen and its dendrimeric conjugate in the presence of iontophoresis

Treatment	Plasma concentration of ketoprofen (ng/ml)*	
	1 h	**3 h**
Ketoprofen + Iontophoresis	22.83 ± 1.10	142.11± 6.55
D2 + Iontophoresis	249.25 ± 12.12*	985.49 ± 43.25*

All the values are expressed as Mean±SD, n=6; * significantly different ($p<0.05$) compared to D4+sonophoresis at the respective time intervals.

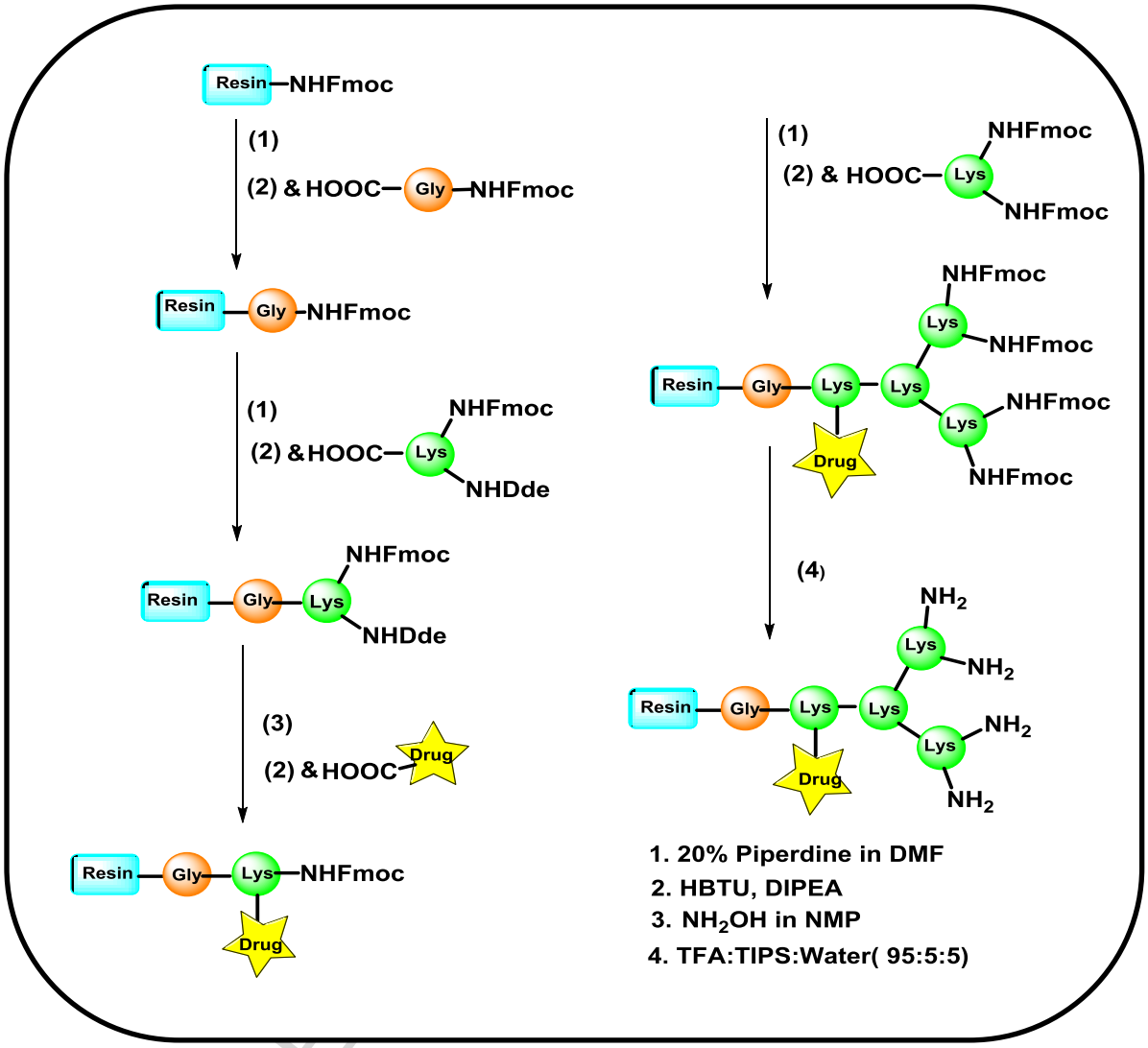
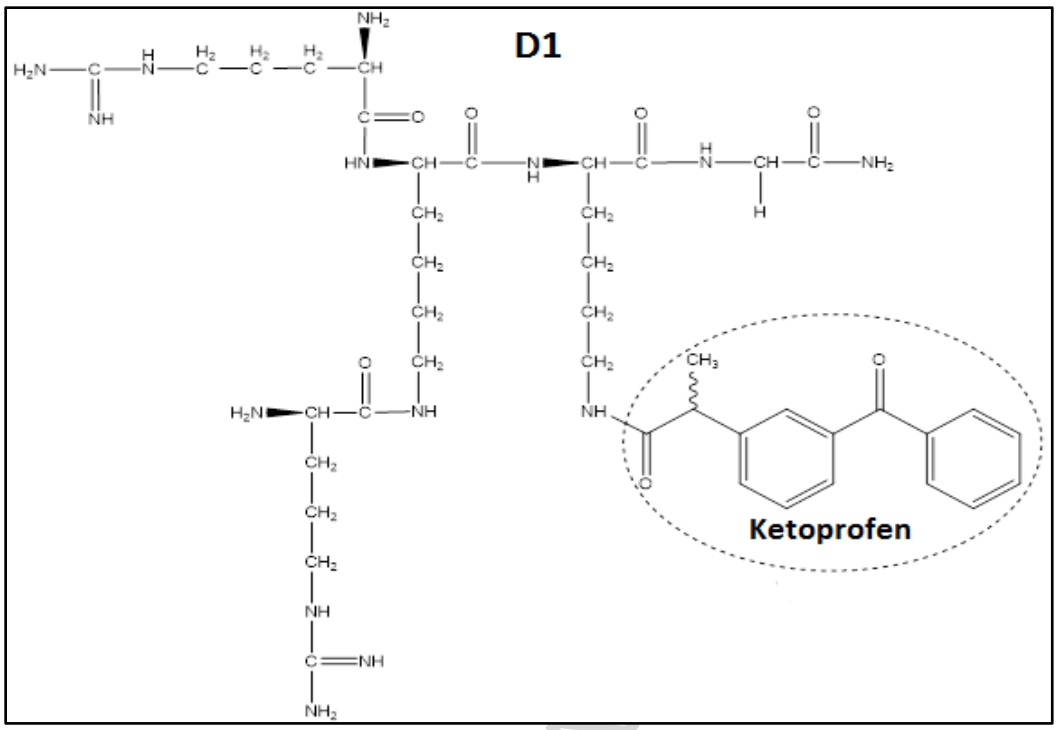
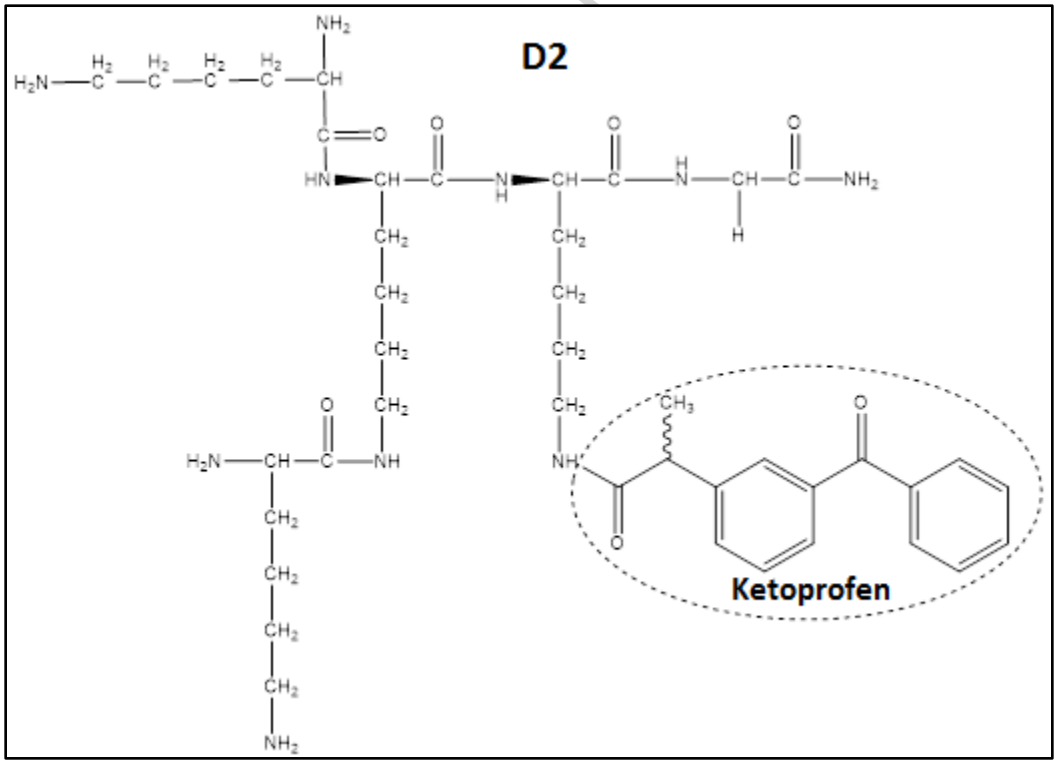


Figure 1. Synthetic scheme towards peptide dendrimeric conjugates of ketoprofen





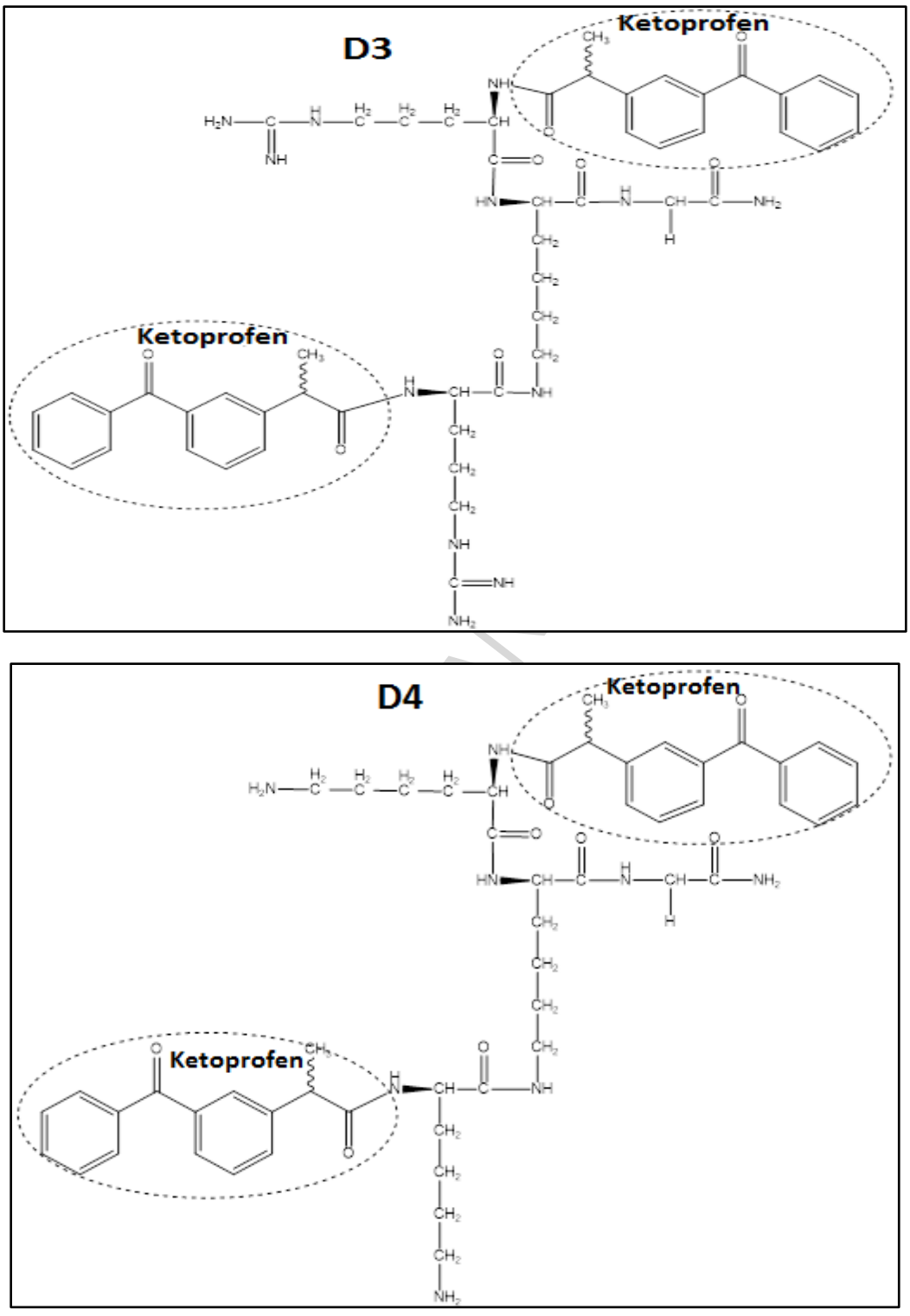


Figure 2. Molecular structures of peptide dendrimeric conjugates of ketoprofen

D1=Gly-Lys(Keto)-Lys-$(Arg)_2$; D2=Gly-Lys(Keto)-Lys$(Lys)_2$; D3=Gly-Lys-$(Arg\text{-}Keto)_2$; D4=Gly-Lys-$(Lys\text{-}Keto)_2$

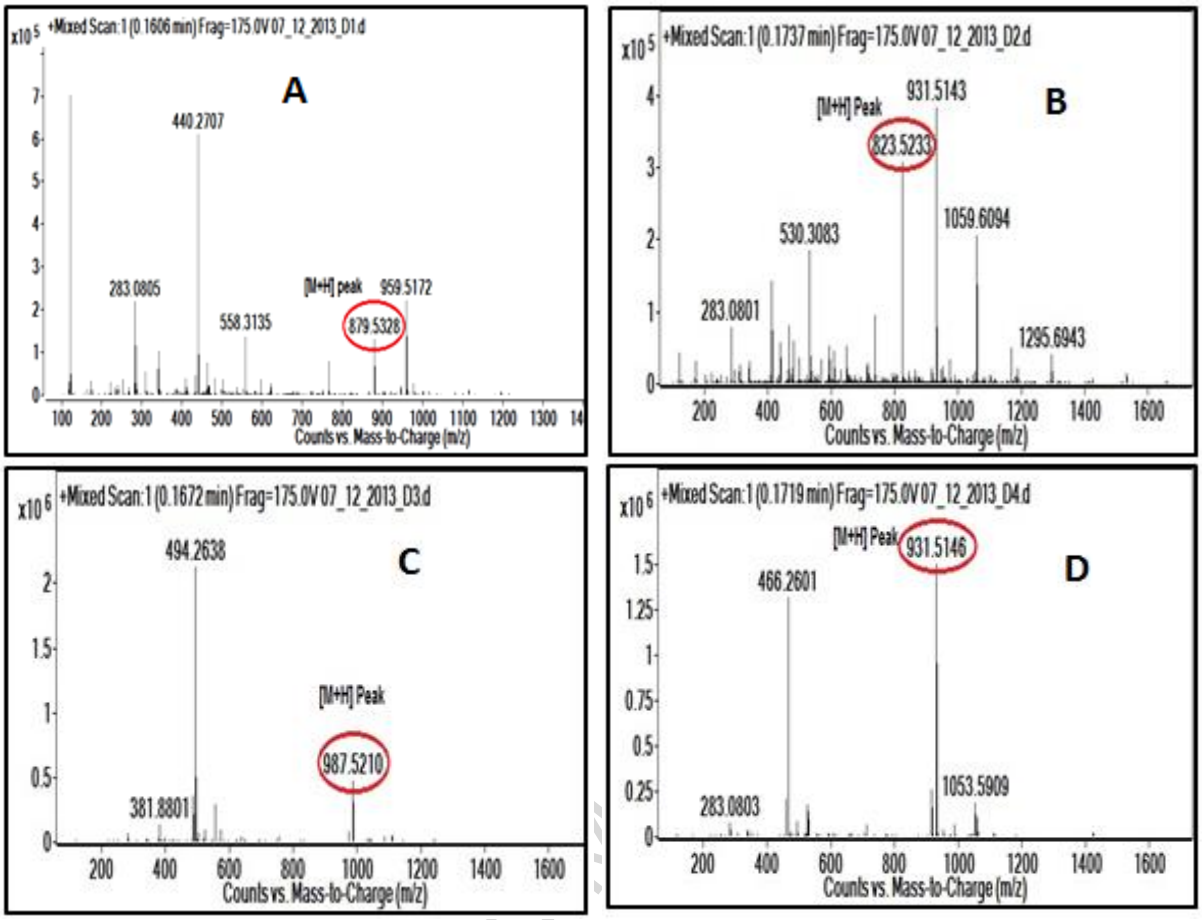


Figure 3. MS Spectra of different ketoprofen-dendrimeric conjugates

A) D1 (Gly-Lys(Keto)-Lys-$(Arg)_2$), B) D2 (Gly-Lys(Keto)-Lys$(Lys)_2$), C) D3(Gly-Lys-(Arg-Keto)$_2$) and D) D4 (Gly-Lys-(Lys-Keto)$_2$)

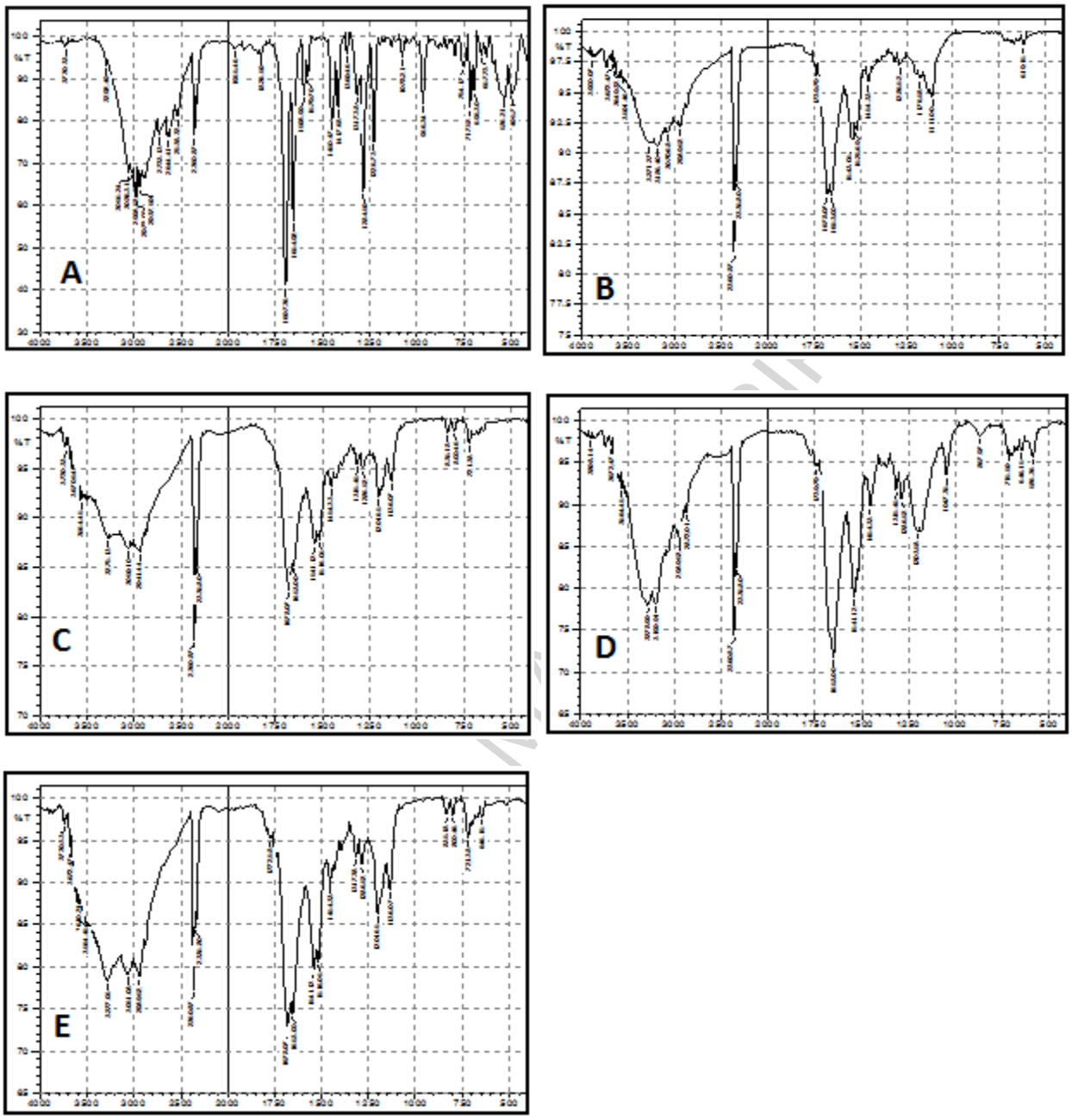


Figure 4. IR spectra of ketoprofen and its dendrimeric conjugates

A) Ketoprofen, B) D1 (Gly-Lys(Keto)-Lys-$(Arg)_2$), C) D2 (Gly-Lys(Keto)-Lys$(Lys)_2$), D) D3(Gly-Lys-(Arg-Keto)$_2$) and E) D4 (Gly-Lys-(Lys-Keto)$_2$)

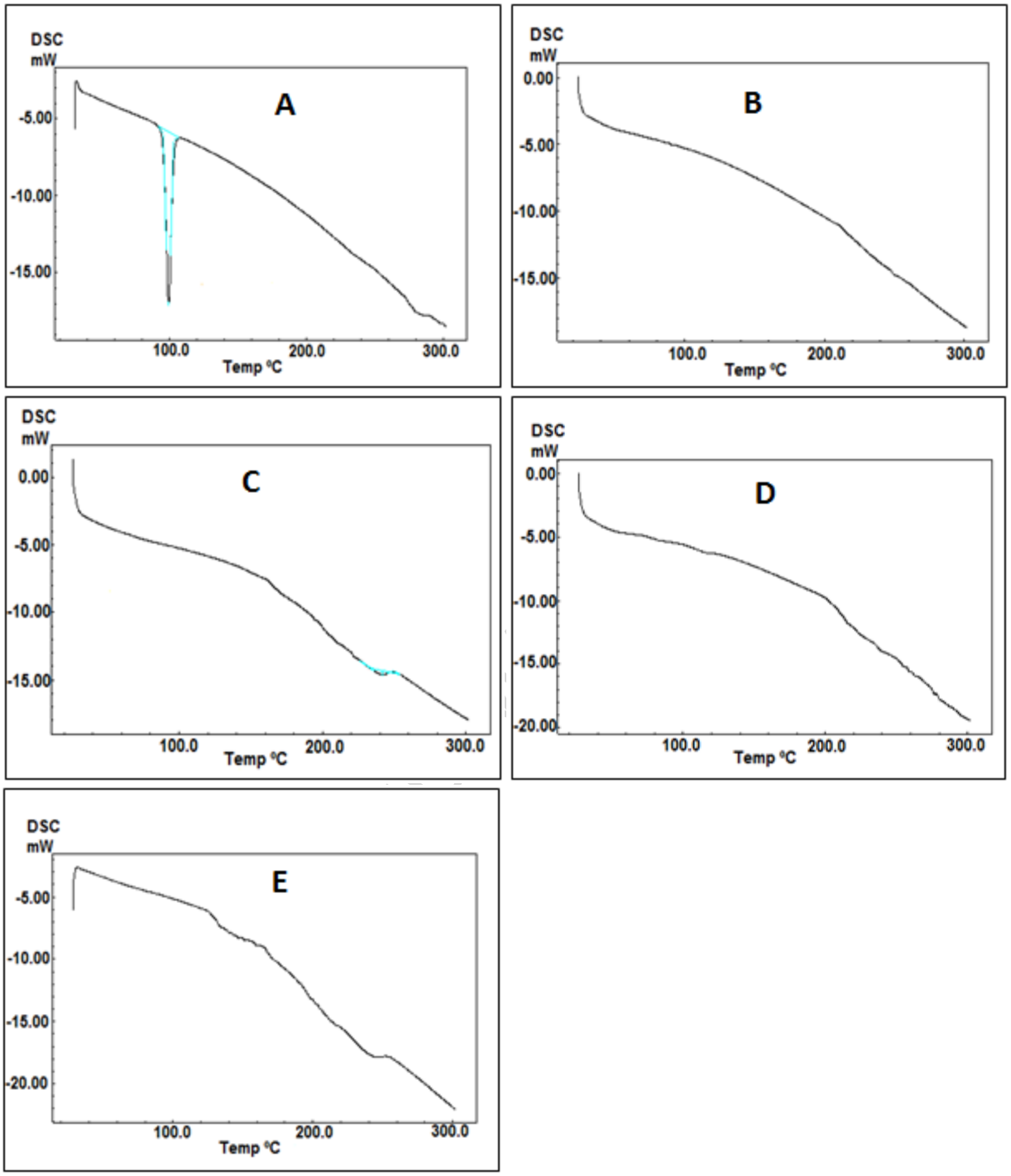


Figure 5. DSC thermograms of pure drug (ketoprofen) ketoprofen and its dendrimeric conjugates (D1, D2, D3 and D4)

A) Ketoprofen, B) D1 (Gly-Lys(Keto)-Lys-$(Arg)_2$), C) D2 (Gly-Lys(Keto)-Lys$(Lys)_2$), D) D3(Gly-Lys-(Arg-Keto)$_2$) and E) D4 (Gly-Lys-(Lys-Keto)$_2$)

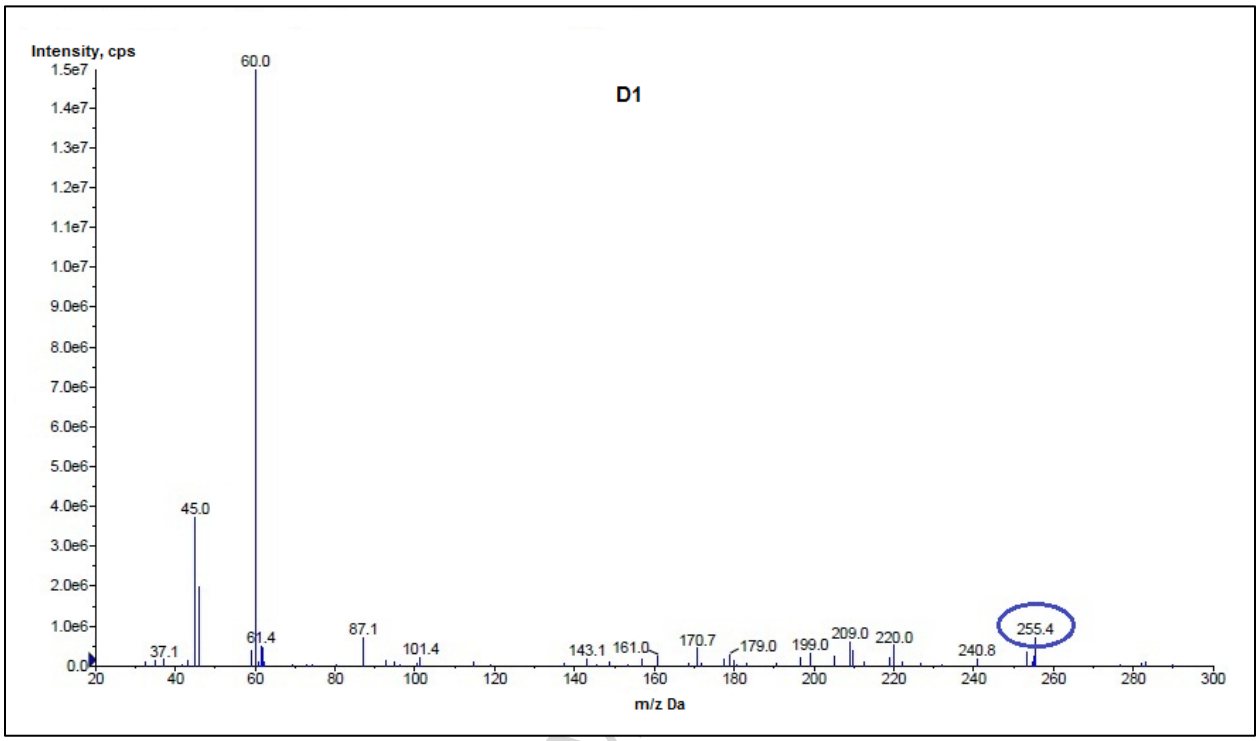


Figure 6. MS image of D1 peptide dendrimeric conjugate indicating molecular ion peak of ketoprofen after treating with trypsin

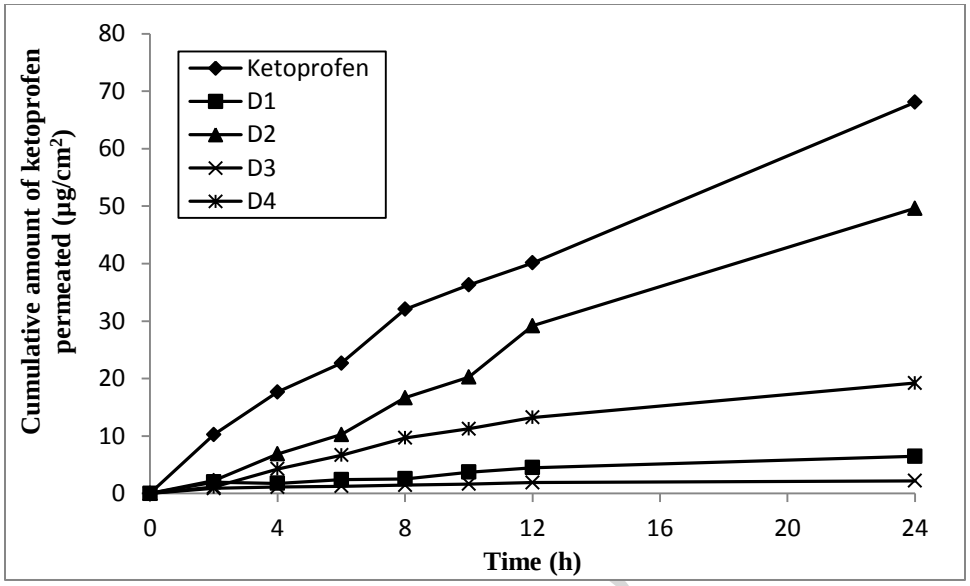


Figure 7. ***In vitro*** **skin permeation profile of ketoprofen and its dendrimeric conjugates in passive diffusion study**

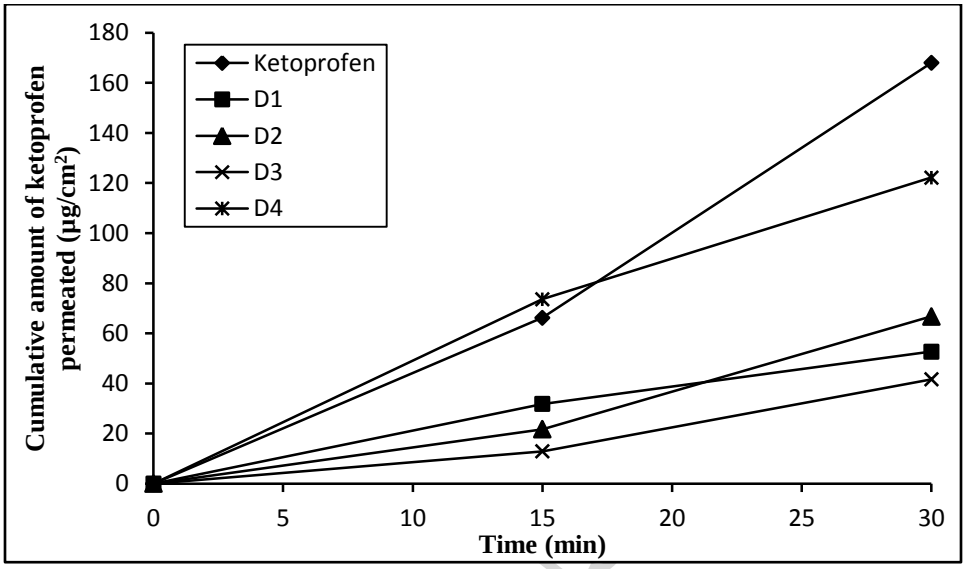


Figure 8. Skin permeation profiles of ketoprofen and its dendrimeric conjugates across the skin with application of ultrasound

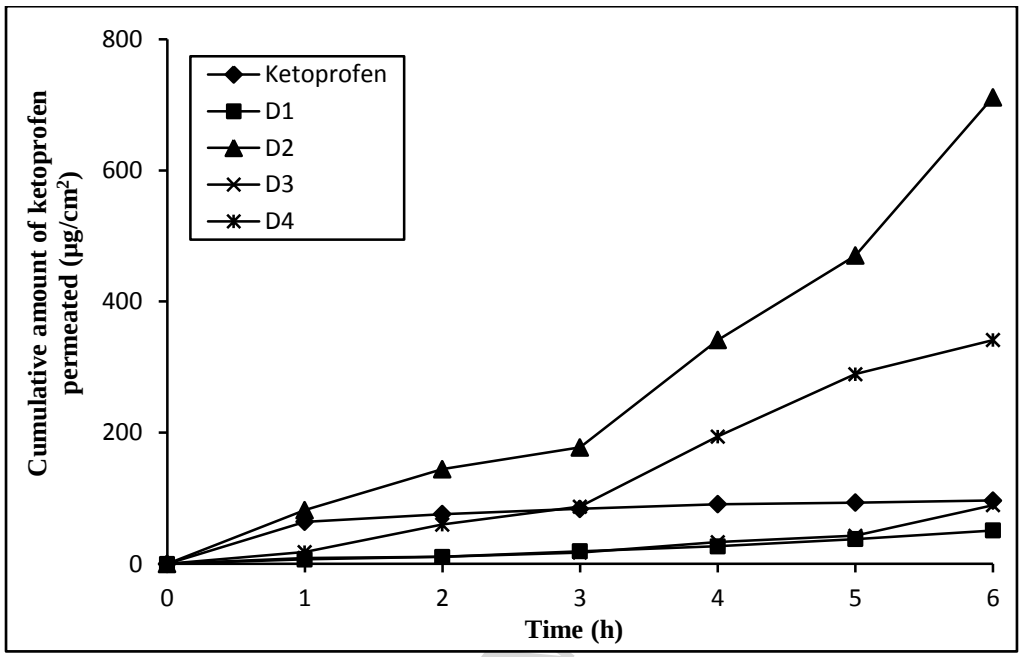


Figure 9. Iontophoretic skin permeation of ketoprofen and its peptide dendrimeric conjugates

Graphical abstract