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FLURBIPROFEN OPHTHALMIC SOLUTIONS TARGETING THE POSTERIOR SEGMENT OF EYE: AN *EX-VIVO* STUDY AND A NOVEL PRODRUG APPROACH ON EXCISED GOAT CORNEA

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ABSTRACT

The current research involves the study of enhancement of trans-corneal permeation Non- Steroidal Anti-Inflammatory Drug, Flurbiprofen via amino acid transporters through excised goat cornea. The prodrug approach by esterification of amino acids followed by conjugation of the amino acid esters with the drug through solvent evaporation technique has been adopted for the synthesis of the physical mixtures of esterified amino acids conjugated with Flurbiprofen. The physical mixtures obtained were characterized by several analytical techniques, purified and were used as active ingredients for preparing the test formulations of Flurbiprofen. In the study, three categories of conjugated physical mixtures of Flurbiprofen with three different amino acids such as L-Arginine, L-Tyrosine and L- valine were synthesized initially by esterification of the amino acids to get methyl and ethyl esters of the amino acids followed by conjugation with Flurbiprofen. The purified products were used as active ingredients for different test formulations of Flurbiprofen. The results obtained from the trans-corneal permeation studies on excised goat cornea during the ex-vivo studies by using a Franz diffusion cell were compared with those of the standard ophthalmic formulations of Flurbiprofen. All results revealed that the permeation of drugs with the amino acid transporters were found to be enhanced through excised goat cornea when compared with the results of the prepared standard formulation of Flurbiprofen.

KEYWORDS: Flurbiprofen, pro-drug, permeation, ex-vivo, diffusion, & amino acid transporters.

INTRODUCTION

Amino acid transporter as drug delivery system

The unique structure of the eye restricts the entry of drug molecules at the required site of action. Ocular administration of drugs is primarily associated with the need to treat ophthalmic disease and is not regarded as a means of gaining systemic drug action. Major classes of drugs used are mydriatics/cycloplegics, anti-inflammatories, anti-infectives, surgical adjuvants, diagnostics, etc. all these are meant for local therapy.[1]Recent advances in topical drug delivery have been made to improve ocular drug contact time and drug delivery, including the development of ointments, gels, liposomes formulations, and various sustained and controlled-release substrates, such as the ocusert, collagen shields, and hydrogel lenses. The development of newer topical drug delivery systems will provide exciting new topical drug therapeutics. The delivery of a therapeutic dose of the drug to the tissue in the posterior segment of the eye, however, remains a significant challenge. Recently, the most appealing approach for improving trans-corneal permeability of hydrophilic moieties appears to be a targeted drug delivery system by means of transporters. One of such approach is by using amino acids transporters.[2]

Drug-amino acids combinations were prepared by initial esterification of amino acids followed by conjugation with the drug and these were targeted to enhance the permeability of drug. Amino acid transporters are responsible for translocation of amino acids from blood to various organs. In eyes, amino acids play an important role in the maintenance of the structural and functional integrity of conjunctiva and retina/RPE.[3] The most effective method for drug targeting is believed to be the amino acids and peptide transporter as this transporter have an enormous range of substrates and direction of transport from epithelium to endothelium providing a possible task in the permeation of substrate molecule. The presences of various amino acid transporters such as

LATI, ATB^{0+} and ASCTI in cornea have been proved. In many kinds of literature, the presence and function of amino acids transporters on the human retina are heavily published. Retinal cells have a basal requirement of amino acids for protein synthesis. Several amino acids neurotransmitters (glutamate, GABA and glycine) and neuroactive amino acids (aspartate, homocysteine acid and taurine) have been identified in the retina.[4] Glutamate which is the major excitatory neurotransmitter is mainly localized on the bipolar cells, retinal ganglion cells and slightly ischemic photoreceptors.[5] In diabetic retinopathy and rhegmatogenous retinal detachment, the vitreal levels of glutamate are mildly elevated. This may be featured by high-affinity excitatory glutamate transport protein that can be utilized in drug delivery.[6] Apparently, there is a study where gene expression level of LAT1 and LAT2 in ARPE-19 cells and finally they have concluded that both LAT1 and LAT2 are involved in L-leucine transport.[7] Overall, these amino acid transport systems could help in the design of prodrugs that are likely to be transported across the retina for better ocular delivery and bioavailability.

L-Lysine is a α-amino acid with the molecular formula of $C_6H_{14}N_2O_2$ and IUPAC name of (2S)-2, 6-diaminohexanoic acid. This amino acid is an essential amino acid, which means that humans cannot synthesize it. It has a molecular weight of 146.18756 g/mol and water solubility of 1000000mg/L at 20°C. L-Phenylalanine is an essential aromatic amino acid that is a precursor of melanin, dopamine, noradrenalin, and thyroxine. IUPAC name is (2S)-2-amino-3-phenylpropanoic acid and a molecular formula of $C_9H_{11}NO_2$. It has a molecular weight of 165.18914g/mol and water solubility of 26900mg/L at 25°C. L-Valine is a branched-chain essential amino acid that has stimulant activity. It promotes muscle growth and tissue repair. It is a precursor in the penicillin biosynthetic pathway. The IUPAC name of L-Valine is (2S)-2-amino-3-methylbutanoic acid and a molecular formula of $C_5H_{11}NO_2$. It has a molecular weight of 117.14634 g/mol and water solubility of 58500mg/L at 25°C.[8]

MATERIALS AND METHODS

Acectic acid, benzene, iodine, calcium chloride, dicyclohexylcarbodiimide, dimethyl formamide, ethanol, ether and tetra hydro furan were obtained from SAS Chemicals Company, Mumbai. Flurbiprofen was obtained as a gift sample from Unisule Pvt. India Ltd., Sonepat. Hydrochloric acid. Hydrxybenzotriazole, I-arginine, l-tyrosine, l-valine, methanol, sodium hydroxide, thionyl chloride were obtained from SD fine chemicals Mumbai.

METHODS

Esterification of amino acids

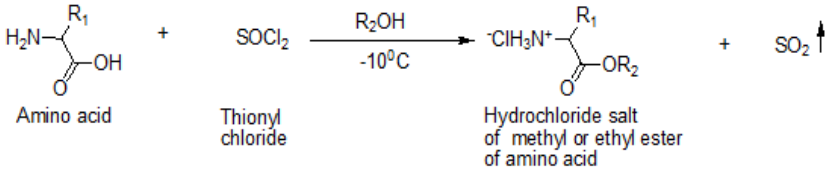
Amino acids used were L-arginine, L-valine and L-tyrosine. Mainly two types of esters (methyl and ethyl esters) using methanol and absolute ethanol were prepared for the three amino acids respectively. The esterification was performed to protect the α-carboxyl group of the amino acids.[9]

Procedure for the synthesis of amino acid esters

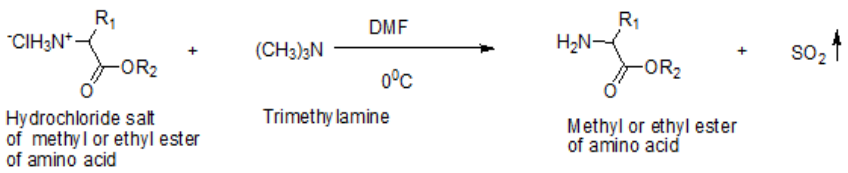
Thionyl chloride was added drop wise, over a period of 30minutes with vigorous stirring, to absolute methanol for methyl esters or ethanol for ethyl esters was placed as required in a round bottom flask equipped with a calcium chloride guard tube and cooled to -10°C. To this solution the respective amino acid was added pinch wise and mixture was stirred for 1 hour at 4°C and refluxed for 5hours. Consumption of thionyl chloride was optimised to 1.6ml for 0.2mole of each amino acid in 100ml methanol/ethanol. The chlorination process was monitored regularly during the reaction to evaluate the need of more thionyl chloride addition. The reaction mixture was concentrated on a rotary evaporator to give the product amino acid ester hydrochloride. The crude product was then washed with ether.[10]

Figure 1.

Step1: Synthesis of hydrochloride salt of methyl or ethyl ester of amino acid



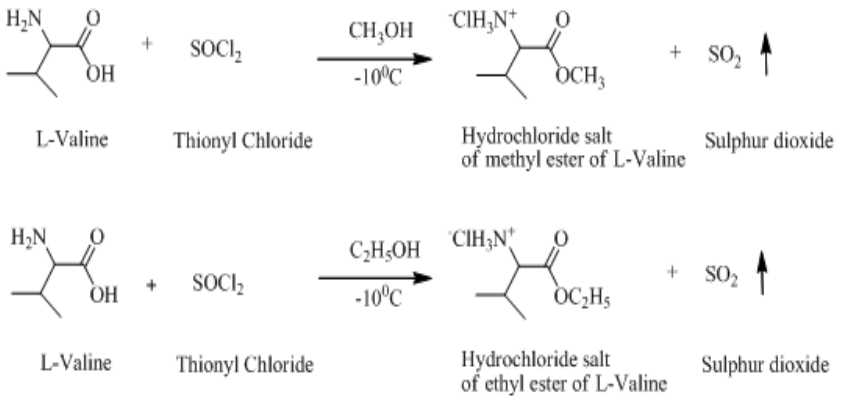
Step2: Synthesis of methyl or ethyl ester of amino acid



DMF: Dimethylformamide.

Proposed synthesis scheme for the formation of methyl and ethyl esters

Figure: 2. Proposed synthesis scheme for the formation of methyl and ethyl esters

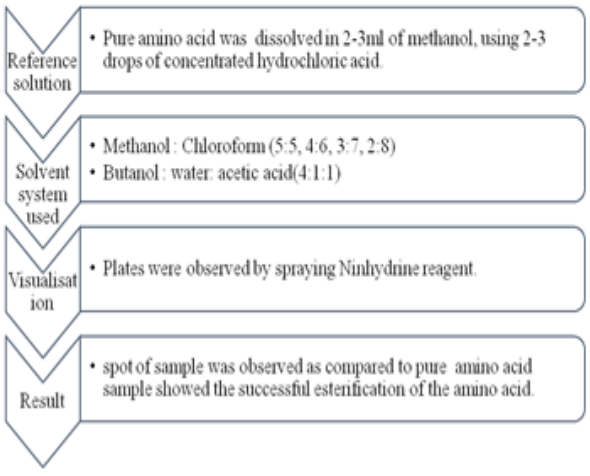


Characterization of synthesized amino acid esters

The esterification process was monitored by performing thin layer chromatography. The major purpose of using TLC was to know whether the reaction is progressing or not. Further esterification was confirmed by the infra-red spectrum of the compounds. To monitor the process of esterification thin layer chromatography was performed during the reaction and retention factor was calculated as:

$$Rf = \frac{\text{Distance travel by the solute from the line of origin}}{\text{Distance travelled by the solvent from the line of origin}}$$

Figure 3. General procedure used for thin layer chromatography



PRODRUG FORMATION

Procedure for synthesis of Flurbiprofen-amino acid prodrugs

Flurbiprofen (0.206g, 1m mole) was dissolved in Tetrahydrofuran, followed by the addition of hydroxybenzotriazole (HOBt) (0.153g, 1mmole) solution in dimethylformamide (DMF). The reaction mixture was stirred for 5 minutes at 0°C. The methyl or ethyl ester of amino acid was added to above reaction mixture at 0°C. To the above mixture was added dicyclohexylcarbodimide (DCC) (0.206g, 1 mmole) was dissolved in THF at 0°C. The reaction mixture was allowed to reach at room temperature and was stirred for 4h. Hydrichloride salt of amino acid ester as well as DCC and HOBT were used in the same molar concentration as that of Flurbiprofen. The dicyclohexylurea (DCU) was filtered off and the filtrate was concentrated on a rotary evaporator.[10]

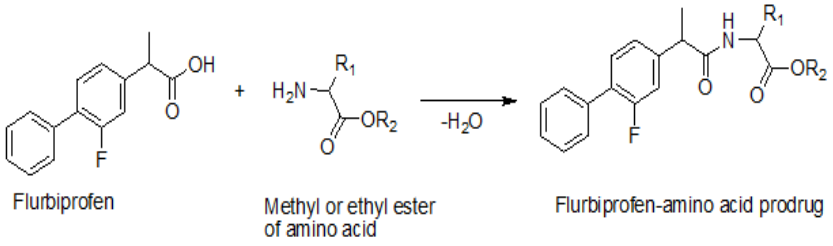


Figure: 4. Synthesis of Flurbiprofen-amino acid prodrug

Work up of the synthesized product

The oily residue obtained after rotary evaporation was dissolved in ethyl acetate, washed with 5% aqueous sodium bicarbonate, brine, 5% citric acid solution and finally with brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude product was purified over silica gel column to get the pure compound.

Characterization of the prodrugs

1. The synthesized compounds were subjected to thin layer chromatography in order to check their purity. The prepared plates of silica gel G adsorbents were dried and activated. The solvent system methanol: acetic acid::ether:: benzene: in the ratio of 1:18:60:20 was used. Iodine vapour was used as detecting agent. All compounds gave single spot.

Schematic representation of pro-drug and its metabolism

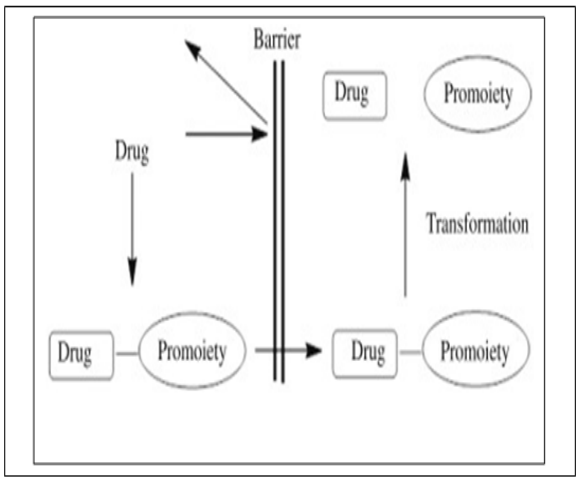


Figure: 5. Schematic representation of prodrug and its metabolism

Ester prodrugs can be converted back to active parent drugs via esterases present in the eyes. Esterases appear to be to be concentrated in the iris-ciliary body, corneal epithelium, retina and optic nerve. Different classes of esterases i.e., acetylcholine esterase, pseudocholine esterase, butyryl-choline esterase and carboxyl esterases are responsible for facile conversion of the ester prodrugs to parent drugs.

Standard curve of Flurbiprofen

Preparation of standard plot of Flurbiprofen in normal saline (0.9 % w/v) pH 7.

Flurbiprofen (100mg) was dissolved in approximately 10ml of 0.1 N NaOH and volume was made up to 100ml with normal saline (0.9%w/v). pH of this solution was adjusted to 7 using 0.1 N HCl and 0.1 N NaOH. This was designated as a stock solution of Flurbiprofen in normal saline having 1000µg/ml concentration. Normal saline was used because

a) It is isotonic with the body fluids and lacrimal fluids and
b) Also, the eye can well tolerate this pH.

The stock solution of the drug (1000µg/ml) was scanned for absorbance in the wavelength range of 200-400nm using UV double beam spectrophotometer (Systronics, Japan) to find out the absorption maxima. The absorption maximum was observed at 247nm. Further dilutions (1µg/ml- 10µg/ml) were made to prepare a standard plot of the drug in normal saline pH 7. The absorbance of each solution was recorded at 247nm (λ max) using double beam UV spectrophotometer.

Preparation of standard plot of Flurbiprofen in homogenized corneal extract

The standard plot of Flurbiprofen was also prepared in the homogenized corneal extract (pH 7.4 in IPBS) for solubility studies. Homogenized corneal extract (pH 7.4 in IPBS) was prepared by homogenizing the cornea in chilled (4^0C) Isotonic Phosphate Buffer Saline (IPBS). Flurbiprofen (100mg) was dissolved in approximately 10ml of 0.1 N NaOH and volume was made up to 100ml with homogenized corneal extract (pH 7.4 in IPBS). pH of this solution was adjusted to 7.4 using 0.1 N HCl and 0.1 N NaOH. This was designated as a stock solution of Flurbiprofen in the homogenized corneal extract (pH 7.4 in IPBS) having 1000µg/ml concentration.

The stock solution of the drug (1000µg/ml) was scanned for absorbance in the wavelength range of 200-400nm using UV Double beam spectrophotometer (Systronics, Japan) to find out the absorption maxima. The absorption maximum was observed at 247nm. Further dilutions (2µg/ml- 14µg/ml) were made to prepare a standard plot of the drug in normal saline pH 7.4. The absorbance of

each solution was read at 247nm (λ max) in a spectrophotometer.

***Ex vivo* solubility study in goat corneal extract**

Solubility study of Flurbiprofen and prodrugs were done in the homogenized corneal extract (pH 7). Freshly excised goat corneas were used. An excess amount of Flurbiprofen was added to 10 ml of the homogenized corneal extract (pH 7) and excess amount (0.5ml, 1mm) of prodrugs was added to 5ml of the homogenized corneal extract (pH 7) and allowed to shake for 24h continuously on a mechanical shaker. After 24h, the volumetric flasks and vials were removed from the mechanical shaker and the saturated solutions were filtered using Whatman filter paper number one. Absorbance was recorded using UV spectrophotometer at 247nm (λ max).

Hydrolysis procedure

Prior to initiation of the experiment the supernatant was equilibrated at 34^0C for about 30minutes. Hydrolysis was initiated by the addition of 0.5ml of a 1mM prodrug solution (Ethyl ester of Valine-Flurbiprofen) to 5ml of the supernatant. The control consisted of 5ml of IPBS instead of the supernatant. Aliquots (2ml) were withdrawn at appropriate time intervals up to 24hrs. Samples were immediately diluted with 2ml chilled methanol to quench the reaction. Subsequently, these were thawed and centrifuged at 12,500rpm for 15 min prior to analysis by UV spectrophotometer. The UV scans show the presence of Flurbiprofen after hydrolytic cleavage.

The UV scan showed the presence of many absorption maxima and also there were no characteristic absorption maxima for the free Flurbiprofen between 220nm to 250nm and hence it is proposed that the prodrug remains intact in the buffer and no breakdown of the prodrug occurs into the control buffer pH 7.

The UV scan showed the characteristic absorption maxima at 247 indicating certainly the cleavage of the prodrug into the supernatant. This may be due to the presence of cleaving enzymes (aminopeptidase) into the supernatant. The investigations also revealed the presence of microproteins into the supernatants of both corneas. As enzymes are proteins there are certainly some enzymes responsible for cleavage. Further, studies are required to confirm the same. Also, the UV scan of the prodrug in control showed the absence of any characteristic absorption peak for free active drug i.e. Flurbiprofen and which is supportive that the control composed of only the buffering salts and no contribution of any type of protein in the control IPBS Buffer. It means, when the prodrug comes in contact with the biological barrier (cornea) will break down to release the active drug i.e.Flurbiprofen. Moreover, literature also reported the presence of aminopeptidase in the homogenate of the conjunctiva and cornea of albino rabbit.

RESULTS

Esterification of the amino acids

The methyl and ethyl esters of all the five amino acids such as L-Arginine, L-Tyrosine, L-Lysine, L-Phenyl alanine and L-Valine were synthesized as shown in the general reactions of chapter three and were used to prepare the corresponding prodrugs of Flurbiprofen and Ketorolac tromethamine respectively.

Synthesis of the prodrugs

The prodrugs of Flurbiprofen and Ketorolac tromethamine were synthesized by coupling them with their corresponding amino acid esters as represented in the schematic illustration shown in the chapter three. All the synthesized prodrugs were subjected for characterization.

Characterization of the synthesized prodrugs of Flurbiprofen

Melting points

Table: 1. Melting points of the synthesized prodrugs of Flurbiprofen

Sl.No.	CODE	PRODRUGS OF FLURBIPROFEN (FPD^s & FPD-A^S)	MELTING POINTS (^{o}C)
1	FPD-1	Flurbiprofen + Methyl ester of L- Arginine	230-232
2	FPD-1A	Flurbiprofen + Ethyl ester of L-Arginine	242-243
3	FPD-2	Flurbiprofen + Methyl ester of L- Tyrosine	149-151
4	FPD-1A	Flurbiprofen + Ethyl ester of L-Tyrosine	160-162
5	FPD-3	Flurbiprofen + Methyl ester of L-Valine	98-100
6	FPD-3A	Flurbiprofen + Ethyl ester of L-Valine	106-108

Table: 2. Rf values of the synthesized pro-drugs of Flurbiprofen

Sl.No.	PRODUCT CODE	PRODRUGS OF FLURBIPROFEN (FPD^s & FPD-A^S)	Rf Values
1	FPD-1	Flurbiprofen + Methyl ester of L- Arginine	0.56
2	FPD-1A	Flurbiprofen + Ethyl ester of L-Arginine	0.51
3	FPD-2	Flurbiprofen + Methyl ester of L- Tyrosine	0.82
4	FPD-2A	Flurbiprofen + Ethyl ester of L-Tyrosine	0.76
5	FPD-3	Flurbiprofen + Methyl ester of L-Valine	0.85
6	FPD-3A	Flurbiprofen + Ethyl ester of L-Valine	0.77

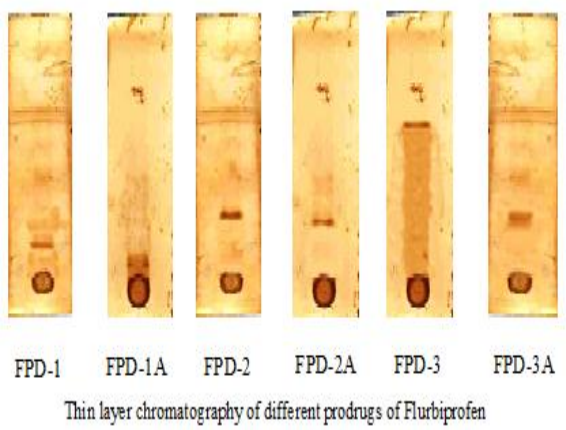


Figure: 6. Thin layer chromatography of the synthesized prodrugs of Flurbiprofen

FTIR Spectra of the synthesized prodrugs of Flurbiprofen

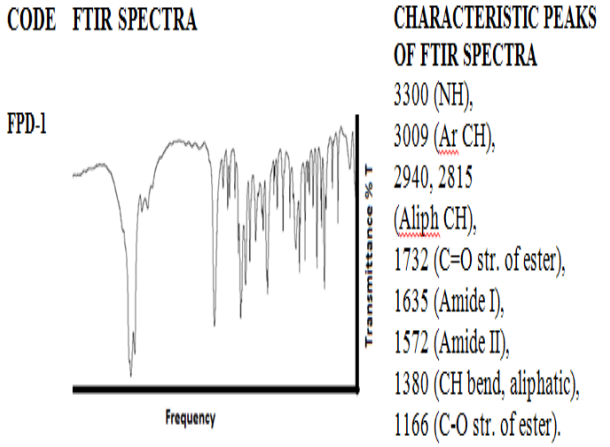


Figure: 7. FTIR Spectra of synthesized prodrug of Flurbiprofen with Methyl ester of L-Arginine.

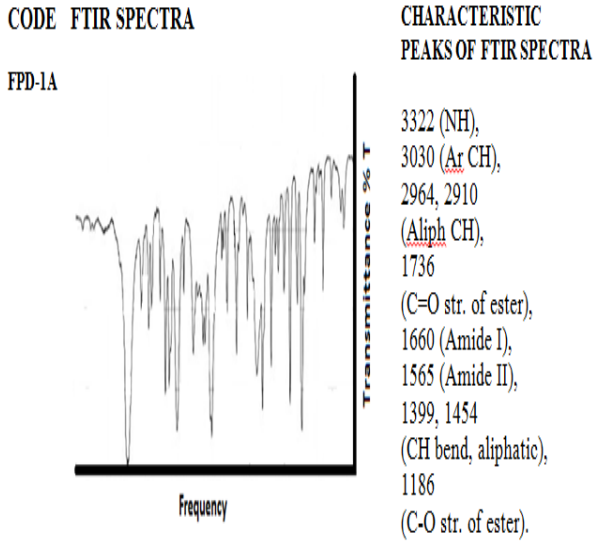


Figure: 8. FTIR Spectra of synthesized prodrug of Flurbiprofen with Ethyl ester of L-Arginine.

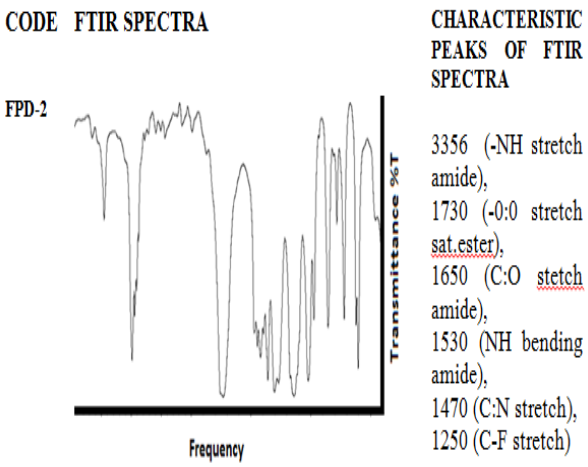


Figure: 9. FTIR Spectra of synthesized prodrug of Flurbiprofen with Methyl ester of L-Tyrosine.

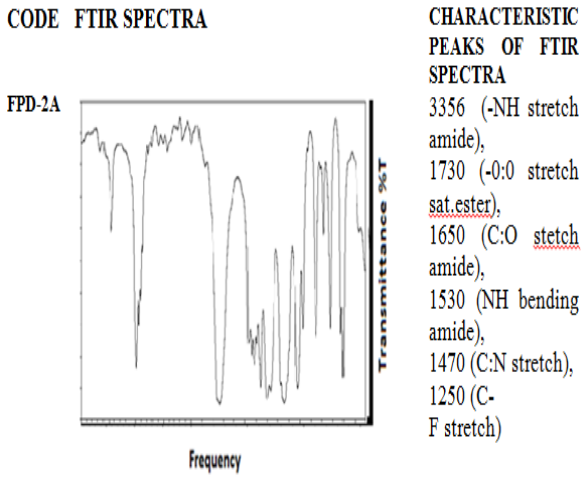


Figure: 10. FTIR Spectra of synthesized prodrug of Flurbiprofen with Ethyl ester of L-Tyrosine.

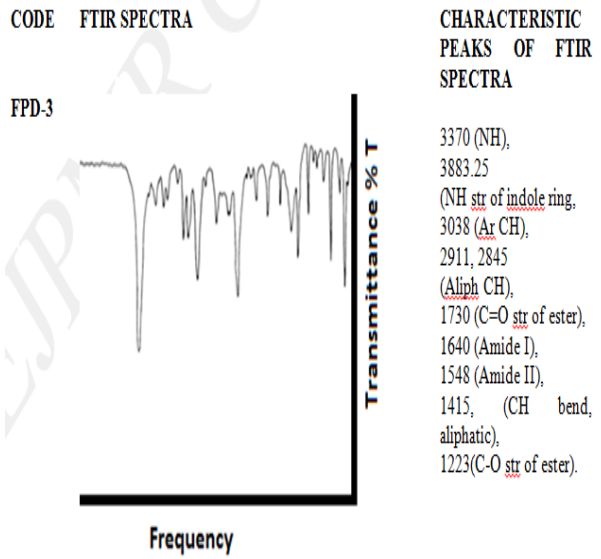


Figure: 11. FTIR Spectra of synthesized prodrug of Flurbiprofen with Methyl ester of L-Valine.

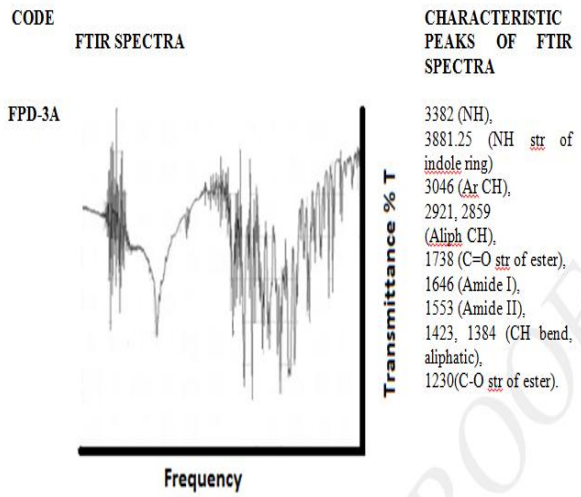


Figure: 12. FTIR Spectra of synthesized prodrug of Flurbiprofen with Ethyl ester of L-Valine.

Spectroscopy of the synthesized prodrugs of Flurbiprofen

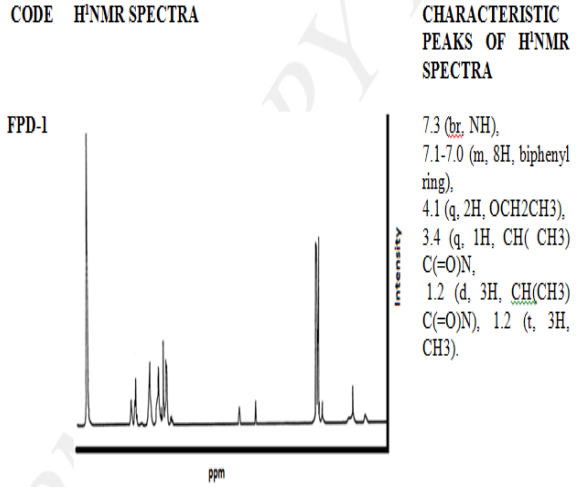


Figure: 13. H[1]NMR Spectra of synthesized prodrug of Flurbiprofen with Methyl ester of L-Arginine.

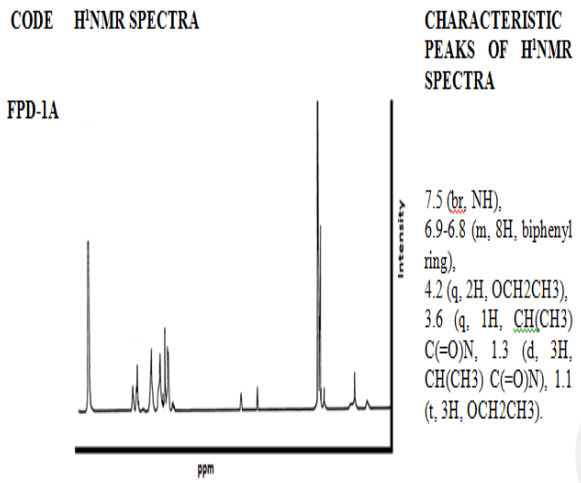


Figure: 14. H[1]NMR Spectra of synthesized prodrug of Flurbiprofen with Ethyl ester of L-Arginine.

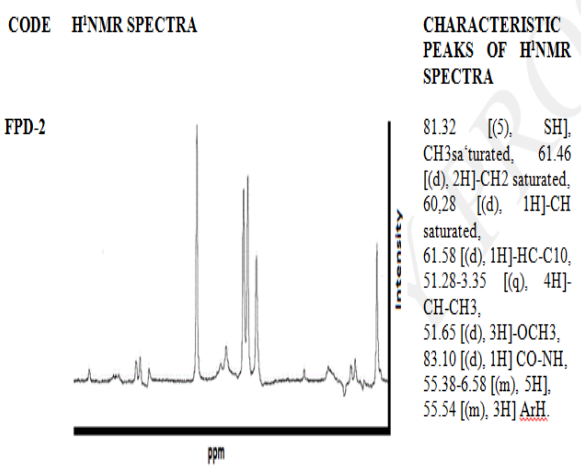


Figure: 15. H[1]NMR Spectra of synthesized prodrug of Flurbiprofen with Methyl ester of L-Tyrosine.

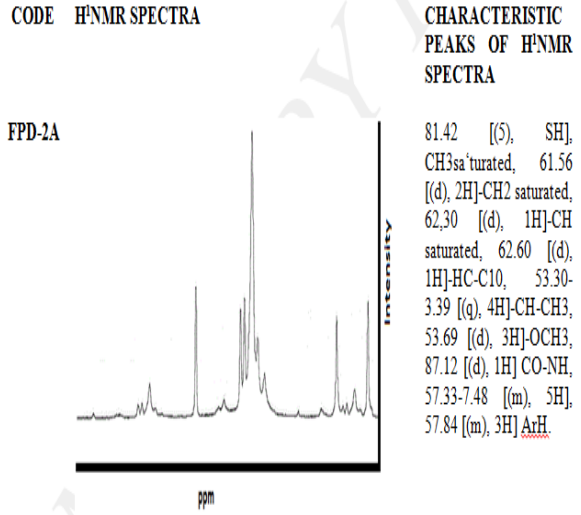


Figure: 16. H[1]NMR Spectra of synthesized prodrug of Flurbiprofen with Ethyl ester of L-Tyrosine.

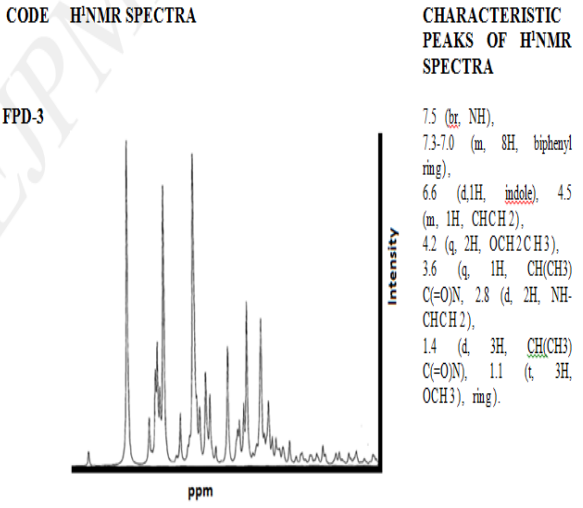


Figure: 17. H[1]NMR Spectra of synthesized prodrug of Flurbiprofen with Methyl ester of L-Lysine.

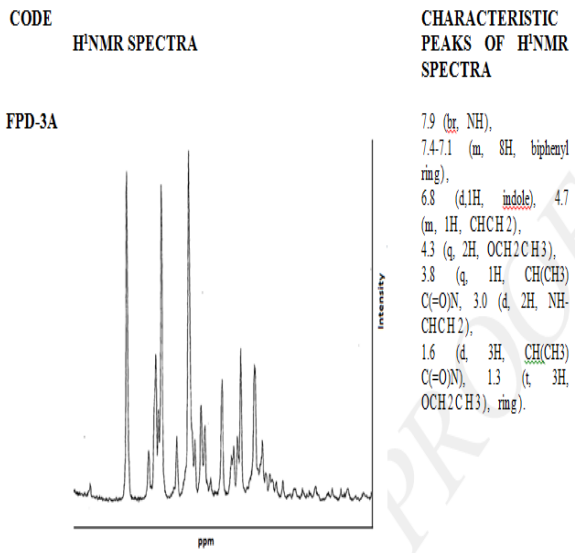


Figure: 18. H[1]NMR Spectra of synthesized prodrug of Flurbiprofen with Ethyl ester of L-Lysine.

MASS spectroscopy of the synthesized prodrugs of Flurbiprofen

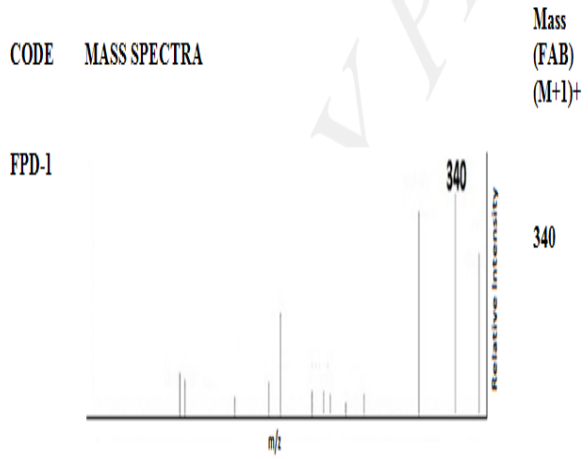


Figure: 19.MASS Spectra of synthesized prodrug of Flurbiprofen with methyl ester of L-Arginine.

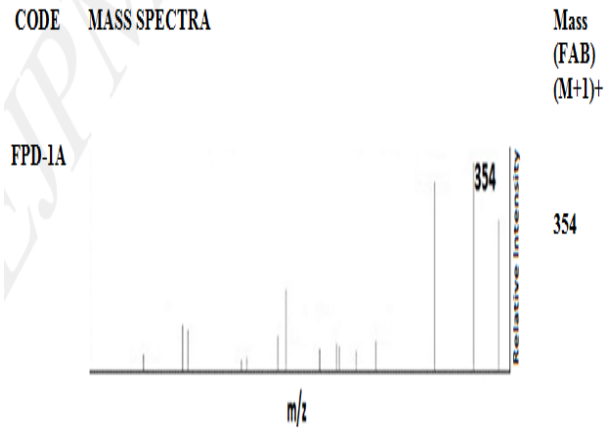


Figure: 20.MASS Spectra of synthesized prodrug of Flurbiprofen with ethyl ester of L-Arginine.

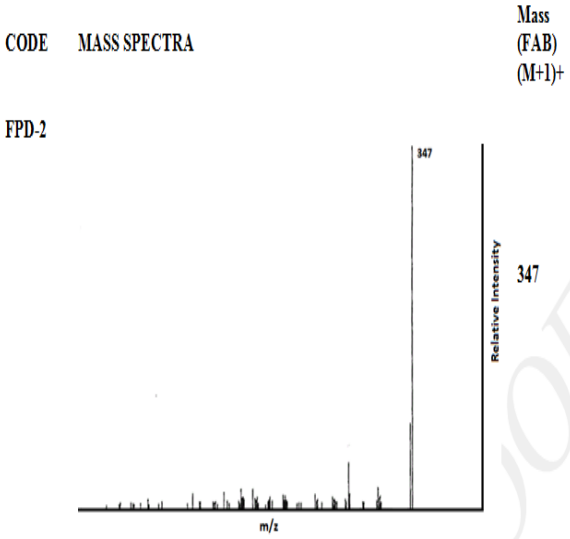


Figure: 21. MASS Spectra of synthesized prodrug of Flurbiprofen with methyl ester of L-Tyrosine.

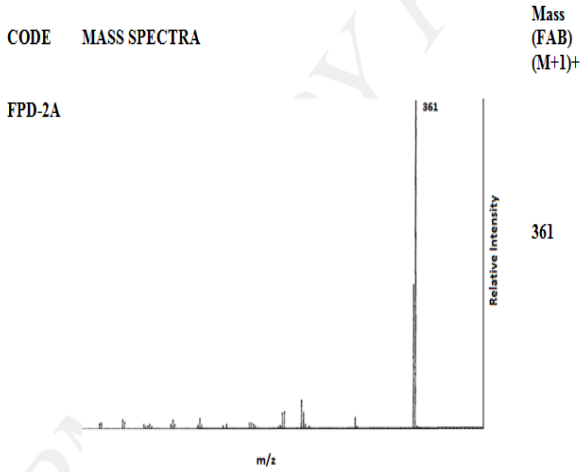


Figure: 22. MASS Spectra of synthesized prodrug of Flurbiprofen with ethyl ester of L-Tyrosine.

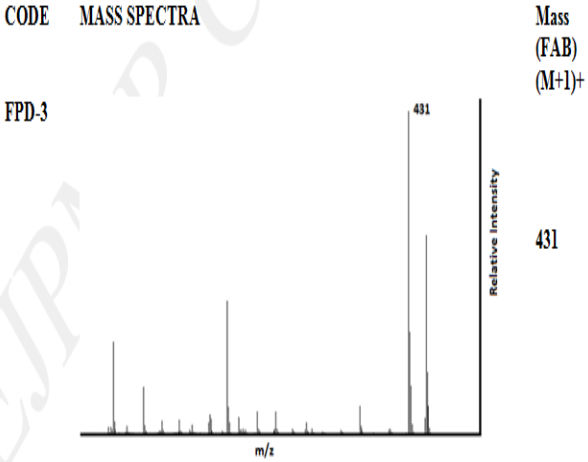


Figure: 23. MASS Spectra of synthesized prodrug of Flurbiprofen with methyl ester of L-Valiine.

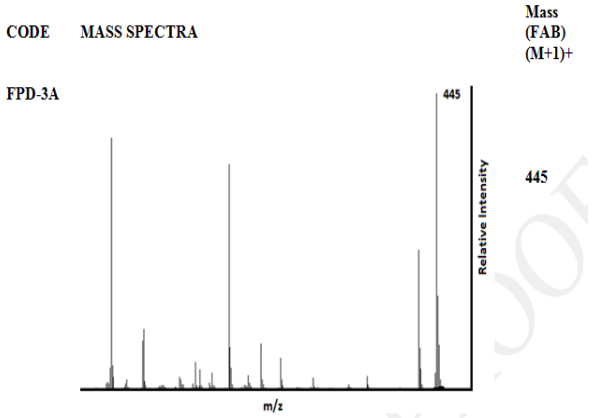


Figure: 24. MASS Spectra of synthesized prodrug of Flurbiprofen with ethyl ester of L-Valiine.

Ex-vivo trans-corneal permeation study of the synthesized prodrugs of Flurbiprofen Standard calibration curve of different concentrations of Flurbiprofen in Normal saline (0.9%w/v NaCl) at pH 7.

The standard plot of different concentrations of Flurbiprofen in Normal saline (0.9%w/v NaCl) at pH 7 against the absorbance was found to be linear in the concentration range of 1-10µg/ml.

Table: 3. Absorbance values for standard calibration curve of Flurbiprofen in Normal saline (0.9%w/v NaCl) pH 7 at λmax 247 nm.

Sl.No.	Concentration (µg/ml)	Absorbance
1	0	0.000
2	1	0.042
3	2	0.086
4	3	0.150
5	4	0.178
6	5	0.212
7	6	0.260
8	7	0.296
9	8	0.357
10	9	0.400
11	10	0.434
Slope = 0.0435, R^2 = 0.9971		

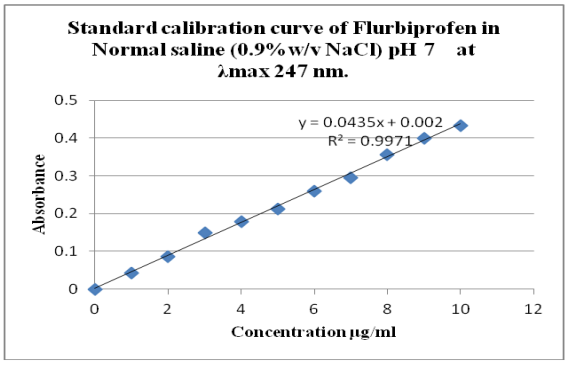


Figure: 25. Standard calibration curve of Flurbiprofen in Normal saline (0.9%w/v NaCl) pH 7 at λmax 247 nm.

Standard calibration curve of Flurbiprofen in homogenised corneal extract in IPBS at pH 7.

The standard plot of different concentrations of Flurbiprofen in in homogenised corneal extract in IPBS (pH 7.) against absorbance was found to be linear in the concentration range of 2-16µg/ml.

Table: 4. Absorbance values for standard calibration curve of Flurbiprofen in homogenised corneal extract in IPBS at pH 7.

Sl. No.	Conc. (µg/ml)	Absorbance
1	0	0
2	2	0.034
3	4	0.078
4	6	0.123
5	8	0.158
6	10	0.198
7	12	0.242
8	14	0.289
9	16	0.324
Slope=0.0205, R^2=0.9992		

Inference: Curve plotted was linear with R^2 value 0.9992 which showed that there is a proportional increase in the concentration with increase in absorbance and this standard curve was further used for solubility studies.

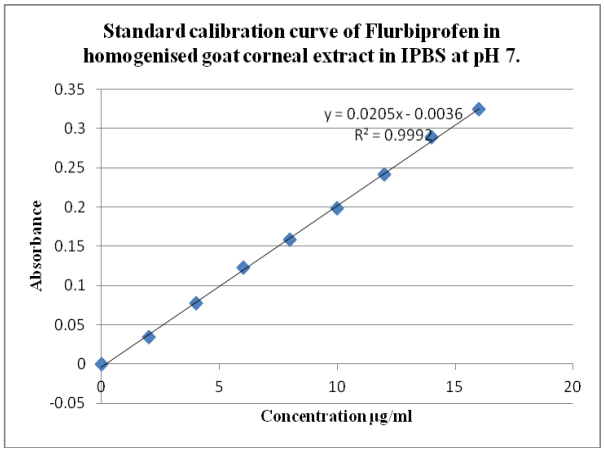


Figure: 26 Standard calibration curve of Flurbiprofen in homogenised corneal extract in IPBS at pH 7.

Table: 5 Absorbance values for standard calibration curve of Flurbiprofen in methanol

Sl.No.	Concentration (µg/ml)	Absorbance
1	0	0.000
2	2 µg/ml	0.158
3	4 µg/ml	0.296
4	6 µg/ml	0.472
5	8 µg/ml	0.594
6	10 µg/ml	0.729
7	12 µg/ml	0.912
Slope=0.0746, R^2=0.9982		

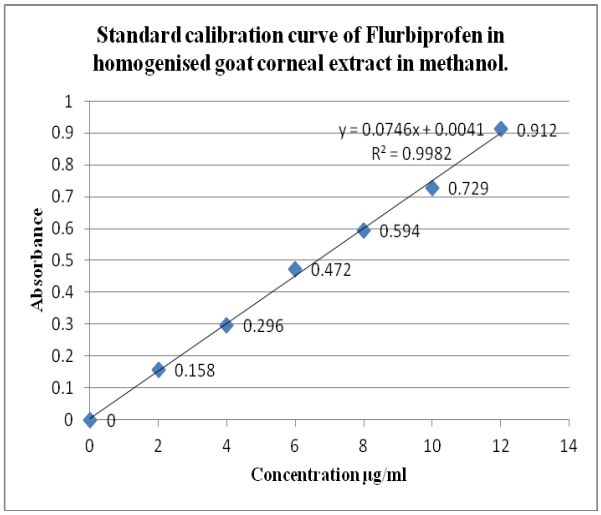


Figure: 27. Standard calibration curve of Flurbiprofen in homogenised corneal extract in methanol.

Table: 6. Absorbance values for standard calibration curve of Flurbiprofen in simulated tear fluid

Sl.N0.	Concentration (µg/ml)	Absorbance
1	0	0.000
2	2 µg/ml	0.102
3	4 µg/ml	0.180
4	6 µg/ml	0.286
5	8 µg/ml	0.380
6	10 µg/ml	0.474
7	12 µg/ml	0.572
8	14 µg/ml	0.670
9	16 µg/ml	0.772
10	18 µg/ml	0.870
Slope=0.0482, R^2=0.9997		

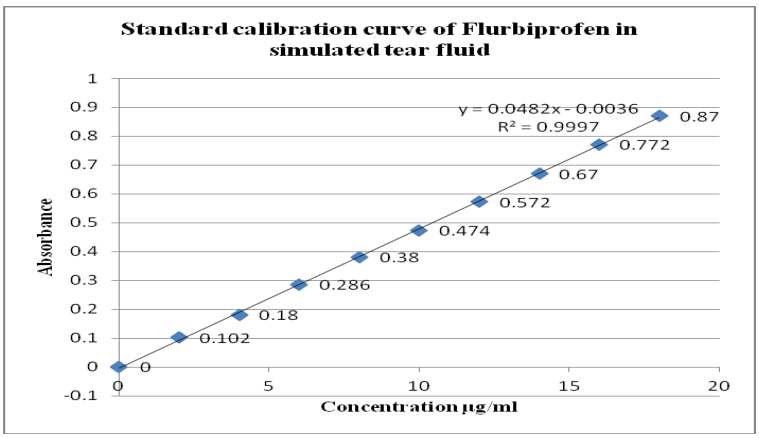


Figure: 28. Standard plot of Flurbiprofen in simulated tear fluid

Table: 7. Effect of concentration of Flurbiprofen aqueous solution (pH 7) on permeation of drug through excised goat cornea

Concentration (w/v)	% Permeation (120 min)	Amount permeated (mg) (12 min)	% Corneal hydration (120 min)
0.03%	0.6470±0.2102	0.1941±0.1051	82.54±1.783
0.05%	0.3857±0.2211	0.19285±0.2211	80.62±2.733
0.10%	0.3228±0.0394	0.3228±0.05910	77.84±1.475
0.15%	0.2247±0.0099	0.33705±0.0199	77.82±1.476

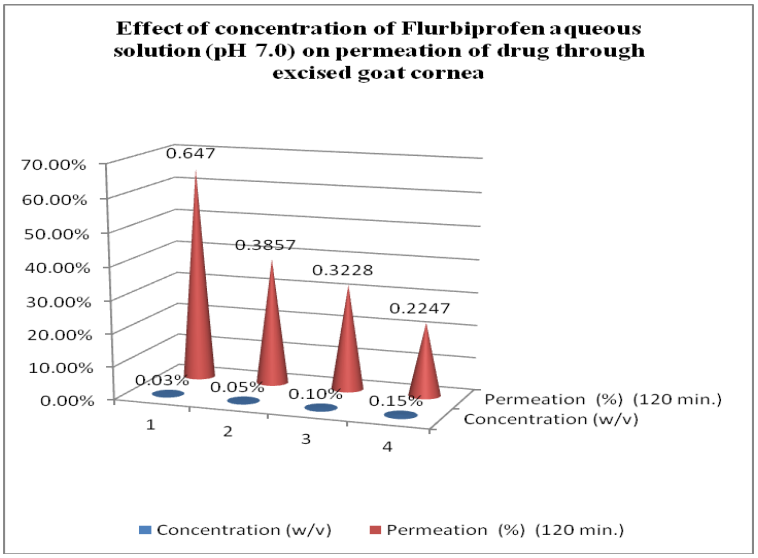


Figure: 29. Effect of concentration of Flurbiprofen aqueous solution (pH 7) on permeation of drug through excised goat cornea

Table: 8. Effect of pH of Flurbiprofen aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea

pH	% Permeation (120 min)	Amount permeated (mg) (12 min)	% Corneal hydration (120 min)
6.8	0.4458±0.0842	0.1321±0.0421	80.87±0.546
7.0	0.6470±0.2102	0.1941±0.1051	79.82±1.212
7.2	0.5645±0.0332	0.1693±0.0166	78.33±2.028
7.4	0.4619±0.1727	0.1385±0.0863	82.54±1.783
7.6	0.3322±0.1727	0.0966±0.0264	81.24±1.246
(Values are mean ± SE of 3 corneas in each group)			

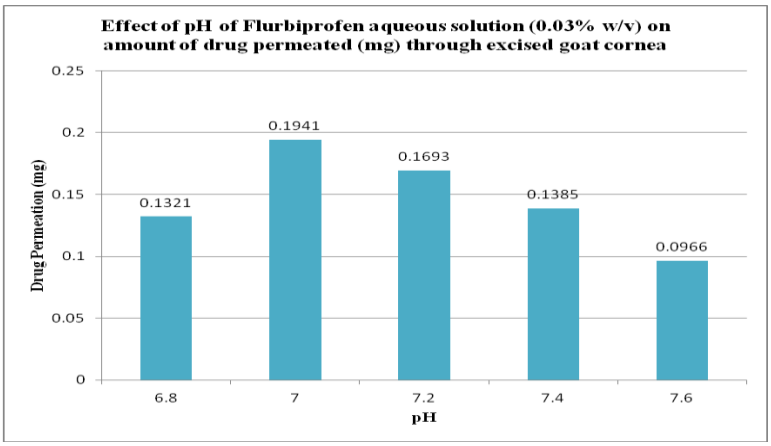


Figure: 30. Effect of pH of Flurbiprofen aqueous solution (0.03% w/v) on amount of drug (mg) permeated through excised goat cornea

Table: 9. Effect of pH of Flurbiprofen pro-drug (FPD-1) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea

pH	Permeation (%) (120 min.)	Amount permeated (mg)(120 min.)	Corneal hydration (%) (120 min.)
6.8	0.6484±0.0424	0.1945±0.0012	68.60±1.24
7.0	1.4050±0.0640	0.4215±0.0323	72.98±0.62
7.2	0.7246±0.0443	0.2173±0.0018	74.34±0.25
7.4	0.5016±0.1201	0.1504±0.0013	71.97±1.04
7.6	0.4328±0.2402	0.1298±0.0010	80.47±1.04
(Values are mean ± SE of 3 corneas in each group)			

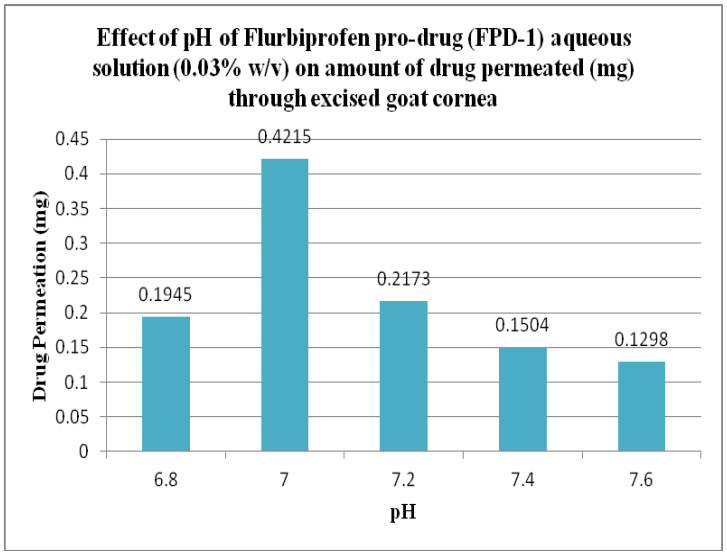


Figure: **31**. **Effect of pH of Flurbiprofen prodrug (FPD-1) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea**

Table: 10. Effect of pH of Flurbiprofen prodrug (FPD-1A) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea

pH	Permeation (%) (120 min.)	Amount permeated (mg)(120 min.)	Corneal hydration (%) (120 min.)
6.8	0.6244±0.0121	0.1873±0.0012	69.60±2.24
7.0	1.3164±0.0270	0.3949±0.0323	72.98±0.62
7.2	0.71428±0.0213	0.2142±0.0018	74.34±0.25
7.4	0.5116±0.0214	0.1504±0.0013	72.97±1.46
7.6	0.4241±0.0262	0.1272±0.0010	84.47±1.04
(Values are mean ± SE of 3 corneas in each group)			

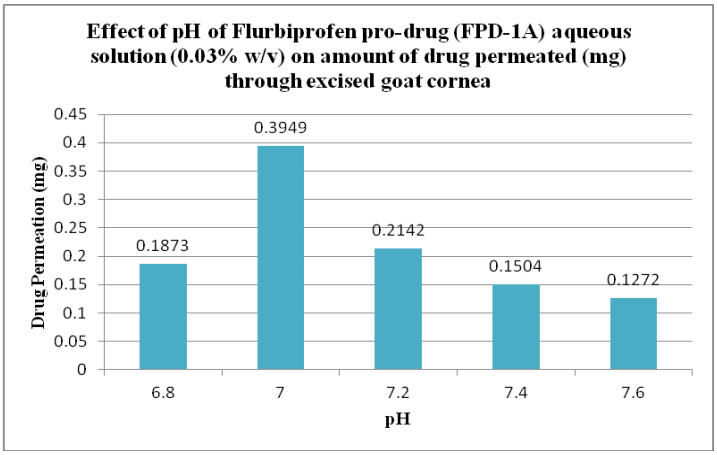


Figure: 32. **Effect of pH of Flurbiprofen prodrug (FPD-1A) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea**

Table: 11. Effect of pH of Flurbiprofen prodrug (FPD-2) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea

pH	Permeation (%) (120 min.)	Amount permeated (mg)(120 min.)	Corneal hydration (%) (120 min.)
6.8	0.6024±0.0424	0.1945±0.0213	68.86±2.14
7.0	1.2422±0.0640	0.3726±0.1024	72.98±0.62
7.2	0.7024±0.0572	0.2107±0.1426	74.34±0.25
7.4	0.4824±0.2132	0.1447±0.0221	73.97±2.04
7.6	0.4042±0.1632	0.1212±0.0247	82.44±1.02
(Values are mean ± SE of 3 corneas in each group)			

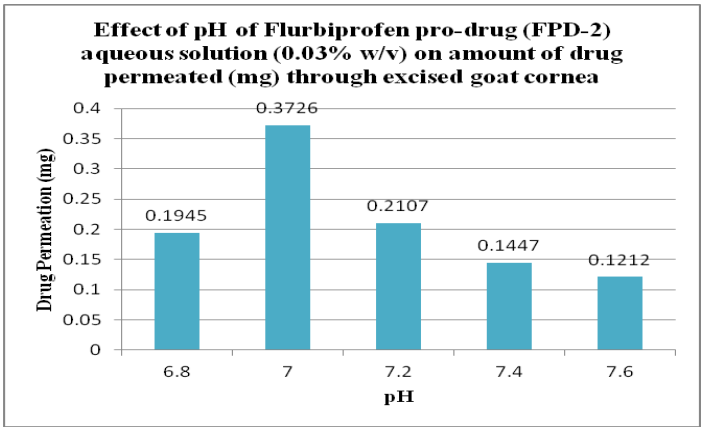


Figure: 33. Effect of pH of Flurbiprofen prodrug (FPD-2) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea

Effect of pH of Flurbiprofen prodrug (FPD-2A) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea

Table: 12. Effect of pH of Flurbiprofen prodrug (FPD-2A) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea

pH	Permeation (%) (120 min.)	Amount permeated (mg)(120 min.)	Corneal hydration (%) (120 min.)
6.8	0.6126±0.0214	0.1837±0.0142	69.56±1.46
7.0	1.144±0.0110	0.3432±0.0224	72.66±0.42
7.2	0.7228±0.0417	0.2168±0.0126	74.44±0.12
7.4	0.5014±0.0412	0.1504±0.1143	76.97±1.66
7.6	0.4022±0.0212	0.1206±0.0014	81.22±1.64
(Values are mean ± SE of 3 corneas in each group)			

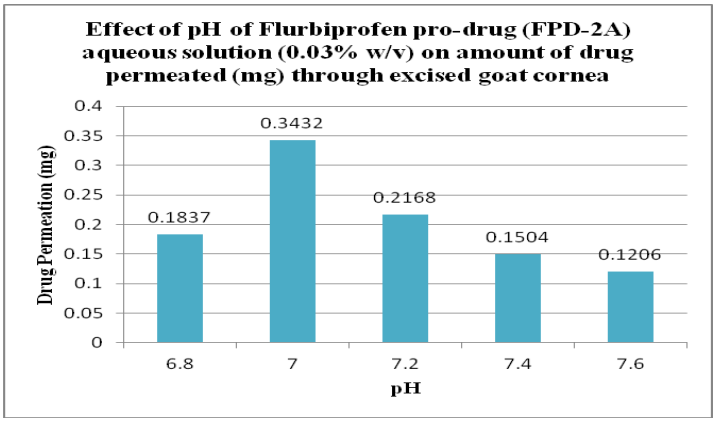


Figure: 34. Effect of pH of Flurbiprofen prodrug (FPD-2A) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea

Effect of pH of Flurbiprofen prodrug (FPD-3) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea

Table: 13. Effect of pH of Flurbiprofen prodrug (FPD-3) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea

pH	Permeation (%) (120 min.)	Amount permeated (mg)(120 min.)	Corneal hydration (%) (120 min.)
6.8	0.6884±0.0424	0.2065±0.0024	71.68±1.24
7.0	1.6248±0.0640	0.4874±0.0212	70.66±0.24
7.2	0.9246±0.0443	0.2773±0.0224	76.32±0.22
7.4	0.7016±0.1201	0.2104±0.0012	75.97±1.02
7.6	0.6328±0.2402	0.1898±0.0010	82.22±1.22
(Values are mean ± SE of 3 corneas in each group)			

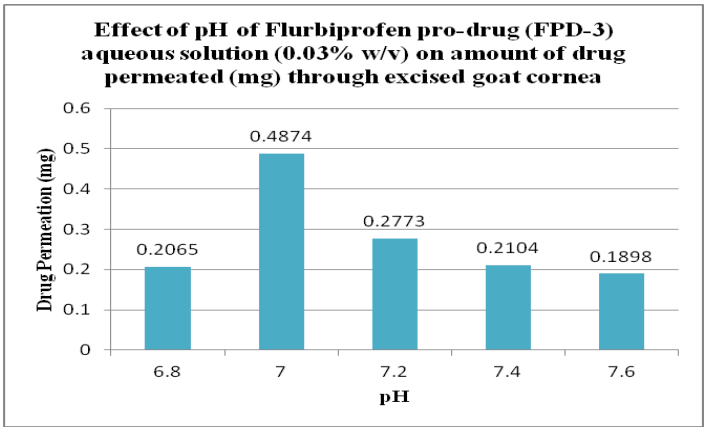


Figure: 35. Effect of pH of Flurbiprofen prodrug (FPD-3) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea

Effect of pH of Flurbiprofen prodrug (FPD-3A) aqueous solution (0.05%) on permeation of drug through excised goat cornea

Table: 14. Effect of pH of Flurbiprofen prodrug (FPD-3A) aqueous solution (0.05%) on permeation of drug through excised goat cornea

pH	Permeation (%) (120 min.)	Amount permeated (mg)(120 min.)	Corneal hydration (%) (120 min.)
6.8	0.6442±0.0212	0.19326±0.0246	72.48±1.32
7.0	1.4224±0.0422	0.4267±0.0212	71.22±0.44
7.2	0.9024±0.0221	0.2707±0.0112	76.46±0.46
7.4	0.7214±0.0120	0.2164±0.0232	78.92±1.62
7.6	0.6143±0.1244	0.1842±0.0281	81.33±1.13
(Values are mean ± SE of 3 corneas in each group)			

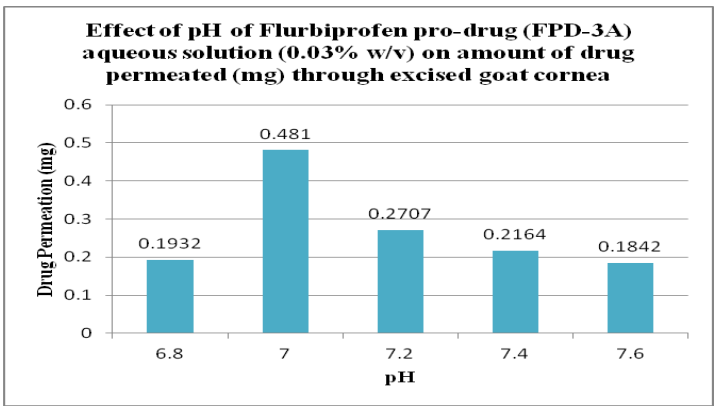


Figure: 36. Effect of pH of Flurbiprofen prodrug (FPD-3A) aqueous solution (0.05%) on permeation of drug through excised goat cornea

Comparison of percentage permeability of Flurbiprofen solution (0.03%w/v) with percentage permeability of the solutions of Flurbiprofen pro-drugs (FPD-1 and FPD-1A) at pH 6.8, 7.0, 7.2 , 7.4 and 7.6.

Table: 15. Comparison of percentage permeability of Flurbiprofen solution (0.03%w/v) with percentage permeability of the solutions of Flurbiprofen pro-drugs FPD-1 and FPD-1A at pH 6.8, 7.0, 7.2. 7.4 and 7.6.

pH	% Permeability of Flurbiprofen	% Permeability of FPD-1	% Permeability of FPD-1A
6.8	0.4458±0.084	0.6484±0.0424	0.6244±0.0121
7.0	0.6470±0.2102	1.4050±0.0640	1.3164±0.0270
7.2	0.5645±0.0332	0.7246±0.0443	0.71428±0.0213
7.4	0.4619±0.1727	0.5016±0.1201	0.5116±0.0214
7.6	0.3322±0.1727	0.4328±0.2402	0.4241±0.0262

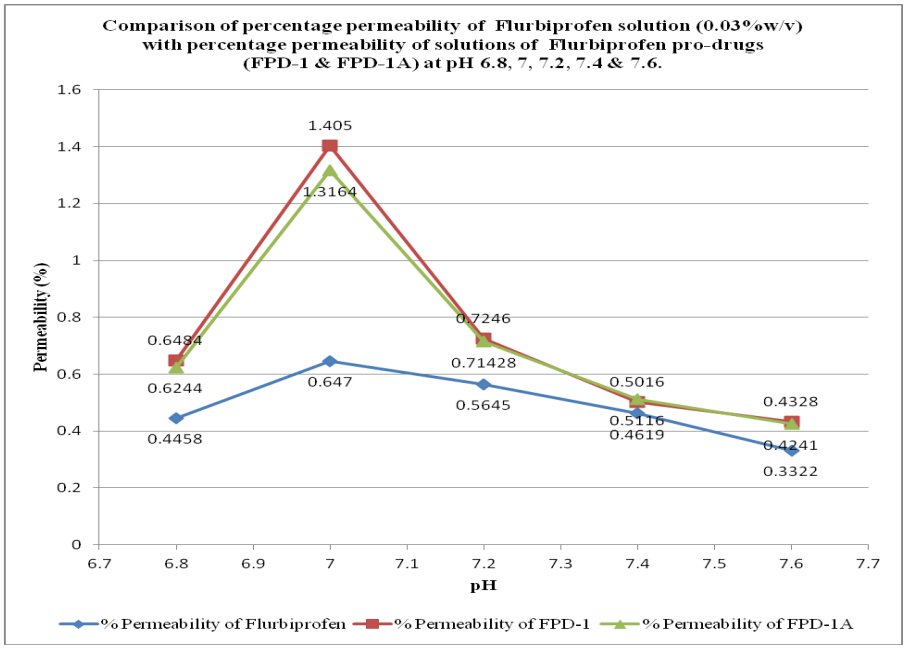


Figure: 37. Comparison of percentage permeability of Flurbiprofen solution (0.03%w/v) with percentage permeability of the solutions of Flurbiprofen pro-drugs (FPD-1 and FPD-1A) at pH 6.8, 7.0, 7.2 , 7.4 and 7.6.

Comparison of percentage permeability of Flurbiprofen solution (0.03%w/v) with percentage permeability of the solutions of Flurbiprofen pro-drugs (FPD-2 and FPD-2A) at pH 6.8, 7.0, 7.2 , 7.4 and 7.6.

Table: 16. Comparison of percentage permeability of Flurbiprofen solution (0.03%w/v) with percentage permeability of the solutions of Flurbiprofen pro-drugs FPD-2 and FPD-2A at pH 6.8, 7.0, 7.2. 7.4 and 7.6.

pH	% Permeability of Flurbiprofen	% Permeability of FPD-2	% Permeability of FPD-2A
6.8	0.4458±0.084	0.6024±0.0424	0.6126±0.0214
7.0	0.670±0.2102	1.2422±0.0640	1.144±0.0110
7.2	0.5645±0.0332	0.7024±0.0572	0.7228±0.0417
7.4	0.4619±0.1727	0.4824±0.2132	0.5014±0.0412
7.6	0.3322±0.1727	0.4042±0.1632	0.4022±0.0212

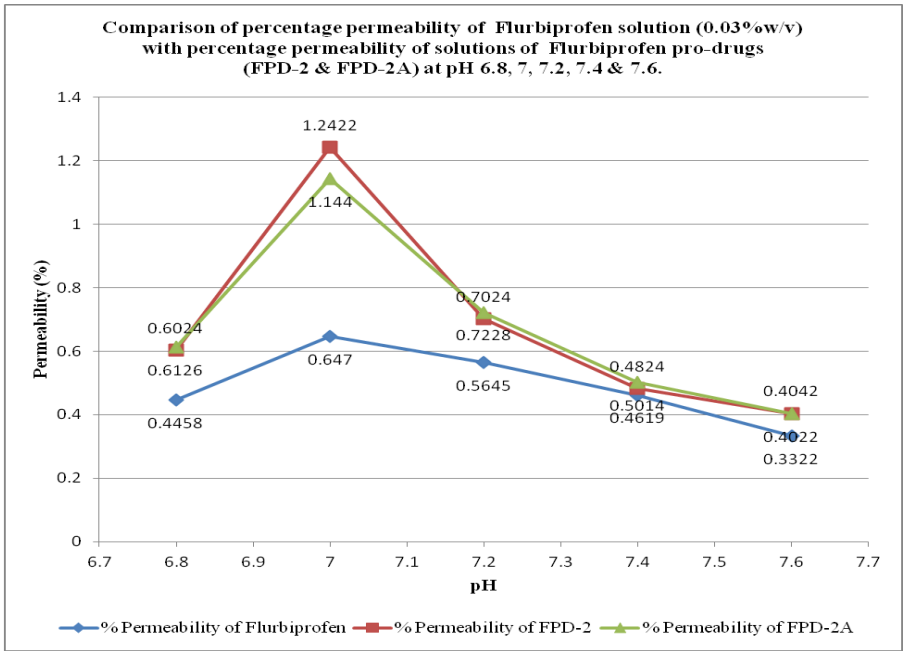


Figure: 38. Comparison of percentage permeability of Flurbiprofen solution (0.03%w/v) with percentage permeability of the solutions of Flurbiprofen pro-drugs (FPD-2 and FPD-2A) at pH 6.8, 7.0, 7.2 , 7.4 and 7.6.

Comparison of percentage permeability of Flurbiprofen solution (0.03%w/v) with percentage permeability of the solutions of Flurbiprofen pro-drugs (FPD-3 and FPD-3A) at pH 6.8, 7.0, 7.2, 7.4 and 7.6.

Table: 17. Comparison of percentage permeability of Flurbiprofen solution (0.03%w/v) with percentage permeability of the solutions of Flurbiprofen pro-drugs FPD-2 and FPD-2A at pH 6.8, 7.0, 7.2. 7.4 and 7.6.

pH	% Permeability of Flurbiprofen	% Permeability of FPD-3	% Permeability of FPD-3A
6.8	0.4458±0.084	0.6884±0.0424	0.6442±0.0212
7.0	0.670±0.2102	1.6248±0.0640	1.4224±0.0422
7.2	0.5645±0.0332	0.9246±0.0443	0.9024±0.0221
7.4	0.4619±0.1727	0.7016±0.1201	0.7214±0.0120
7.6	0.3322±0.1727	0.6328±0.2402	0.6143±0.1244

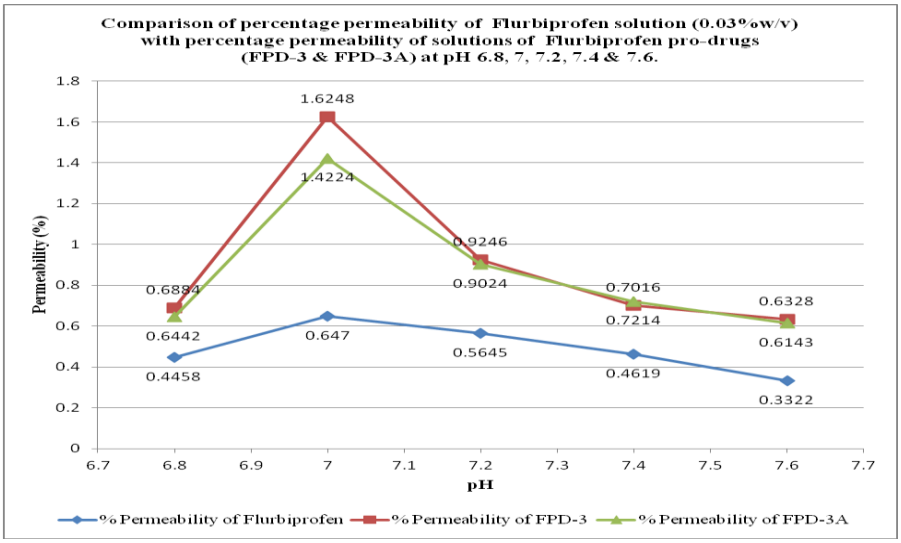


Figure: 39. Comparison of percentage permeability of Flurbiprofen solution (0.03%w/v) with percentage permeability of the solutions of Flurbiprofen pro-drugs (FPD-3 and FPD-3A) at pH 6.8, 7.0, 7.2, 7.4 and 7.6.
Comparison of percentage corneal hydration of Flurbiprofen solution (0.03%w/v) with the corneal hydration of the solutions of Flurbiprofen pro-drugs (FPD-1 and FPD-1A) at pH 6.8, 7.0, 7.2, 7.4 and 7.6.

Table 18.Comparison of percentage corneal hydration of Flurbiprofen solution (0.03%w/v) with the corneal permeation of the solutions of Flurbiprofen pro-drugs FPD-1 and FPD-1A at pH 6.8, 7.0, 7.2. 7.4 and 7.6.

pH	% Corneal hydration of Flurbiprofen	% Corneal hydration of FPD-1	% Corneal hydration of FPD-1A
6.8	80.87±0.546	68.60±1.24	69.60±2.24
7.0	79.8±1.212	72.98±0.62	72.98±0.62
7.2	78.33±2.028	74.34±0.25	74.34±0.25
7.4	82.54±1.783	71.97±1.04	72.97±1.46
7.6	81.24±1.246	80.47±1.04	84.47±1.04

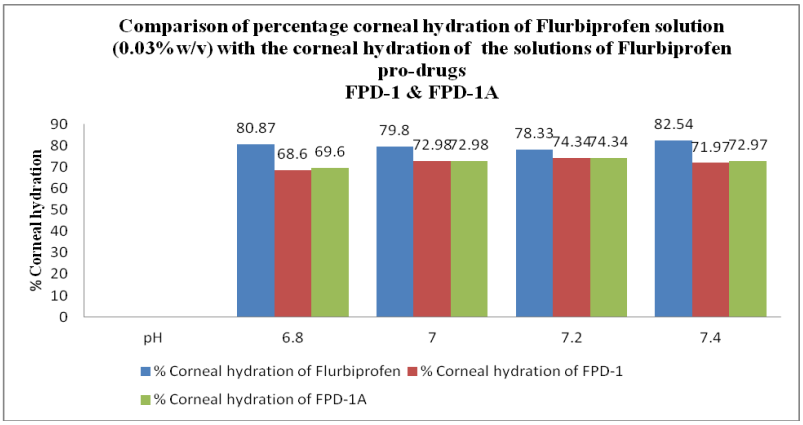


Figure: 40. Comparison of percentage corneal hydration of Flurbiprofen solution (0.03%w/v) with the corneal hydration of the solutions of Flurbiprofen pro-drugs (FPD-1 and FPD-1A) at pH 6.8, 7.0, 7.2, 7.4 and 7.6.

Comparison of percentage corneal hydration of Flurbiprofen solution (0.03%w/v) with the corneal hydration of the solutions of Flurbiprofen pro-drugs (FPD-2 and FPD-2A) at pH 6.8, 7.0, 7.2, 7.4 and 7.6.

Table: 19. Comparison of percentage corneal hydration of Flurbiprofen solution (0.03%w/v) with the corneal permeation of the solutions of Flurbiprofen pro-drugs FPD-2 and FPD-2A at pH 6.8, 7.0, 7.2. 7.4 and 7.6.

pH	% Corneal hydration of Flurbiprofen	% Corneal hydration of FPD-2	% Corneal hydration of FPD-2A
6.8	80.87±0.546	68.86±2.14	69.56±1.46
7.0	79.8±1.212	72.98±0.62	72.66±0.42
7.2	78.33±2.028	74.34±0.25	74.44±0.12
7.4	82.54±1.783	73.97±2.04	76.97±1.66
7.6	81.24±1.246	82.44±1.02	81.22±1.64

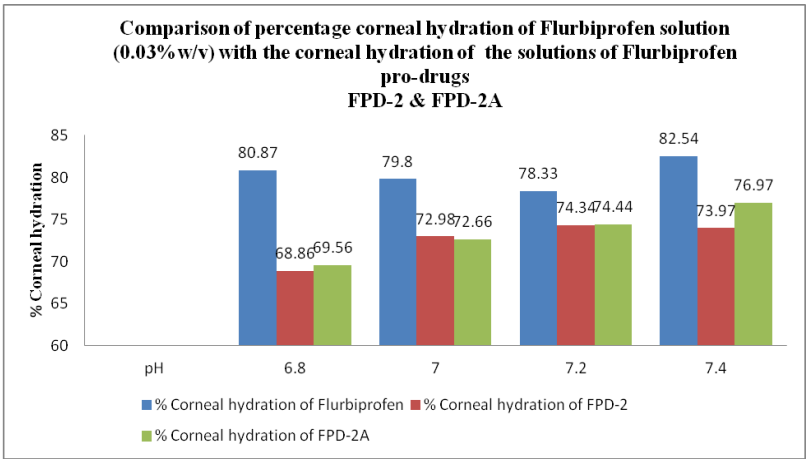


Figure: 41 Comparison of percentage corneal hydration of Flurbiprofen solution (0.03%w/v) with the corneal hydration of the solutions of Flurbiprofen pro-drugs (FPD-2 and FPD-2A) at pH 6.8, 7.0, 7.2, 7.4 and 7.6.
Comparison of percentage corneal hydration of Flurbiprofen solution (0.03%w/v) with the corneal hydration of the solutions of Flurbiprofen pro-drugs (FPD-3 and FPD-3A) at pH 6.8, 7.0, 7.2, 7.4 and 7.6.

Table: 20. Comparison of percentage corneal hydration of Flurbiprofen solution (0.03%w/v) with the corneal permeation of the solutions of Flurbiprofen pro-drugs FPD-3 and FPD-3A at pH 6.8, 7.0, 7.2. 7.4 and 7.6.

pH	% Corneal hydration of Flurbiprofen	% Corneal hydration of FPD-2	% Corneal hydration of FPD-2A
6.8	80.87±0.546	68.86±2.14	69.56±1.46
7.0	79.8±1.212	72.98±0.62	72.66±0.42
7.2	78.33±2.028	74.34±0.25	74.44±0.12
7.4	82.54±1.783	73.97±2.04	76.97±1.66
7.6	81.24±1.246	82.44±1.02	81.22±1.64

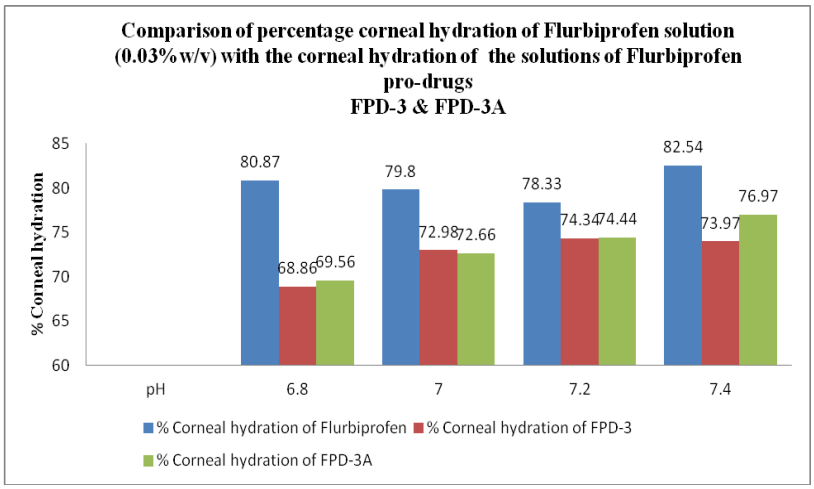


Figure: 42. Comparison of percentage corneal hydration of Flurbiprofen solution (0.03%w/v) with the corneal hydration of the solutions of Flurbiprofen pro-drugs (FPD-3 and FPD-3A) at pH 6.8, 7.0, 7.2, 7.4 and 7.6.

***Ex vivo* solubility study in goat corneal extract**

The drug Flurbiprofen was found to be slightly soluble in the homogenized corneal extract in IPBS (pH 7.4) while both the prodrugs Methyl & Ethyl esters of Valine-Flurbiprofen were found to be sparingly soluble in homogenized corneal extract in IPBS (pH 7.4).

DISCUSSION

All the three amino acids were initially subjected to esterification as explained in Figures 1 and 2, to obtain the methyl and ethyl esters of the corresponding amino acids. The products were characterized by thin layer chromatography as explained in Figure 2. The same were used to get the pro-drugs of Flurbiprofen FPD-1,FPD-1A, FPD-2,FPD-2A, FPD-3 and FPD-3A with corresponding amino acid esters as explained in the Figure 4. The synthesized prodrugs were purified and characterized by determining their melting points which were shown in Table 1. Followed by Thin Layer Chromatography. The Rf values of all the products were shown in Table 2. All the TLC plates were shown in the Figure 5. Several analytical techniques such as FTIR, NMR and Mass spectroscopy were employed to confirm the synthesized pro-drugs. The characteristic peaks and the results of FTIR were shown in Figure 7 to Figure 12. All the characteristic peaks confirmed the synthesized pro-drugs. The NMR spectra of all the pro-drugs were shown in Figure 13 to Figure 18 and all peaks confirmed them. The mass spectra of all the products were shown in Figure 19 to Figure 24 and all were found to be characteristic respectively.

Standard calibration curves of Flurbiprofen were shown in Figure 25,26, 27 and Figure 28 with the absorbance values represented in Table 3, 4, 5 and Table 6 respectively. The effect of concentration of Flurbiprofen aqueous solution was studied with different concentrations such as 0.3,0.5,1.0, and 1.5% w/v and the data obtained was shown in Table 7 and the plot was shown in Figure 29. The results revealed that the solution containing a concentration of 0.03% w/v of Flurbiprofen has maximum (0.647 mg) drug release after 120 minutes of permeation study. The effect of pH of Flurbiprofen aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea was studied with solutions of different pH such as 6.8, 7.0, 7.2, 7.4 and 7.6. The data obtained was shown in Table 8 and the plot was shown in Figure 30. The results revealed that pH 7.0 as best suitable for maximum (0.1941 mg) drug release after 120 minutes. The effect of pH of Flurbiprofen pro-drug (FPD-1) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea was studied with FPD-1 solutions of different pH such as 6.8, 7.0, 7.2, 7.4 and 7.6. The data obtained was shown in Table 9 and the plot was shown in Figure 31. The results revealed that pH 7.0 as best suitable for maximum (0.4215 mg) drug release after 120 minutes. The effect of pH of Flurbiprofen pro-drug (FPD-1A) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea was studied with FPD-1A solutions of different pH such as 6.8, 7.0, 7.2, 7.4 and 7.6. The data obtained was shown in Table 10 and the plot was shown in Figure 32. The results revealed that pH 7.0 as best suitable for maximum (0.3949 mg) drug release after 120 minutes. The effect of pH of Flurbiprofen pro-drug (FPD-2) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea was studied with FPD-2 solutions of different pH such as 6.8, 7.0, 7.2, 7.4 and 7.6. The data obtained was shown in Table 11 and the plot was shown in Figure 33. The results revealed that pH 7.0 as best suitable for maximum (0.3726mg) drug release after 120 minutes. The effect of pH of Flurbiprofen pro-drug (FPD-2A) aqueous solution (0.03%w/v) on permeation of drug through excised goat cornea was studied with FPD-2A solutions of different pH such as 6.8, 7.0, 7.2, 7.4 and 7.6. The data obtained was shown in Table 12 and the plot was shown in Figure 34. The results revealed that pH 7.0 as best suitable for maximum (0.3432 mg) drug release after 120 minutes. The effect of pH of Flurbiprofen pro-drug (FPD-3) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea was studied with FPD-3 solutions of different pH such as 6.8, 7.0, 7.2, 7.4 and 7.6. The data obtained was shown in Table 13 and the plot was shown in Figure 35. The results revealed that pH 7.0 as best suitable for maximum (0.4874 mg) drug release after 120 minutes. The effect of pH of Flurbiprofen pro-drug (FPD-3A) aqueous solution (0.03% w/v) on permeation of drug through excised goat cornea was studied with FPD-3A solutions of different pH such as 6.8, 7.0, 7.2, 7.4 and 7.6. The data obtained was shown in Table 14 and the plot was shown in Figure 36. The results revealed that pH 7.0 as best suitable for maximum (0.4874 mg) drug release after 120 minutes.

The comparison of percentage permeability of Flurbiprofen solution (0.03%w/v) with percentage permeability of the solutions of Flurbiprofen pro-drugs (FPD-1 and FPD-1A) at pH 6.8, 7.0, 7.2 , 7.4 and 7.6 were done and the data was shown in Table 15 with the comparative plot in Figure 37. From the results, it was observed that the pro-drugs FPD-1 and FPD-1A with better and higher drug release among the comparison. Similarly the percentage permeability of Flurbiprofen solution (0.03%w/v) was compared with percentage permeability of the solutions of Flurbiprofen pro-drugs FPD-2, FPD-2A, FPD-3 and FPD-3A and the data were shown in Table 16 and 17 respectively. The plots were shown in Figure 38 and 39. As per the results obtained pH 7.0 is the best suitable for a maximum drug release and when the results of standard Flurbiprofen solution 0.03% were compared with those of the pro-drugs, it was observed that the pro-drugs have the highest drug release in the permeation study. Among all the pro-drugs, the FPD-3 has exhibited the highest (0.4874mg) drug release after 120minutes of the ex-vivo trans-corneal permeation study.

The percentage corneal hydrolysis was determined for all the samples of aqueous solution of Flurbiprofen and the

pro-drugs and compared. The data were represented in Table 18, 19 and Table 20 respectively. The plots were shown in Figure 40,41 and Figure 42 respectively. On comparison it was observed that all the pro-drugs have less percentage corneal permeation when compared to the standard aqueous solution of Flurbiprofen.

CONCLUSION

The methyl and ethyl esters of L-valine were successfully synthesised and was further used for coupling with the pure drug-Flurbiprofen to yield amino acid prodrugs of Flurbiprofen. The prodrugs were characterised by various spectral studies such as FTIR, NMR and Mass spectrometry. All the prodrugs of Flurbiprofen were successful as revealed by the hydrolysis studies using homogenised corneal extract. The *ex- vivo* permeability studies were carried out using excised goat cornea mounted on Franz-diffusion cell and the un-buffered normal saline (0.09%) aqueous test solution of Flurbiprofen was compared with the unbuffered normal saline (0.09%) aqueous test solution of Flurbiprofen prodrugs. All results revealed that the permeation of drugs with all the pro-drugs with amino acid transporters were found to be enhanced through excised goat cornea when compared with the results of the prepared standard ophthalmic formulation of Flurbiprofen on excised goat cornea. Among all the formulated pro-drugs, the methyl and ethyl esters of valine conjugated with flurbiprofen revealed best results and maximum percentage permeability.

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