AN ABSTRACT OF THE THESIS OF

Sumeia M. Mohamed for the degree of Doctor of Philosophy in Pharmaceutical Sciences presented on March 17, 2020.

Title: Transdermal Delivery of Atenolol to Cats.

Abstract approved: _____

John M. Christensen

This thesis describes in-vitro and in-vivo evaluation of a transdermal atenolol formulation developed at Oregon State University, College of Pharmacy. The formulation was prepared from carbomer as a gel base with propylene glycol, glycerol, ethanol, polysorbate 80 and dimethyl-isosorbide (DMI) as mixture of penetration enhancing agents. The effect of the pH on the formulation stability was studied and it was found that pH of 7 can maintain a good stability of atenolol in the formulation. The permeation studies of the invitro phase of this thesis involved 3 different penetration barriers. Synthetic membranes with pore size of 0.45 u was used, followed by cloned human skin which were used to investigate the 6 proposed formulations. Then cloned human epidermis was used to test the three best formulations. Finally, freshly collected cat skin was used for further investigation of the atenolol permeation. Based on the permeation profiles for the different proposed formulations one optimized formulation was chosen which is Formulation (6) with 15% of DMI (Dimethyl isosorbide) and 15% of propylene glycol, 15% glycerol, 10% of ethanol and 5% of polysorbate 80. It was found that the concentration of atenolol 2 hours after application of formulation 6 to

cat skin was 35.5 µg/ml. This correlates to 497 µg penetrating over 2 hours. The therapeutic concentration of atenolol is 260 ng/ml and by considering, atenolol has a volume of distribution of ~1000 ml in cat, therapeutic concentrations of atenolol was considered attainable using this optimized formula. The results of the percent cumulative drug release were examined in accordance to the kinetic models such as Zero-order, First-order, Higuchi equation, Korsmeyer–Peppas equation and Hixson–Crowell equation. In Zero order, R^2 was calculated for each formulation according to the different kinetic models the values of regression coefficient R^2 was 0.9012 for formulation 3, Formulation 5 R^2= 0.9137 and Formulation 6 R^2= 0.92. The graph of the data using Higuchi model was plotted. The cumulative percentage drug released versus square root of time for each formulation through cat skin was not linear and the value of regression coefficients of R^2= 0.607 for formulation 3, and R^2= 0.739 for formulation 5 and formulation 6 value of regression coefficient was R^2= 0.701 were considerably less than the R2 values for the zero-order model. In Hixon-Crowell model, the value of regression coefficient was R^2= 0.541 for formulation 3 and R^2= 0.593 and R^2= 0.653 for formulation 6. The zero-order release model provided the best explanation of drug diffusion through the membranes. The Korsmeyer-Peppas n value for the first phase was 0.8571 for the initial burst flux indicating a fickian diffusion process of a drug solution. The n value for the second flux phase was 0.2616. This indicates that dissolution of the drug from the drug particle occurs before diffusion through the cat skin. Erosion of the drug particle may also be involved in this process.

Three different atenolol formulations were developed to produce an effective formulation. The best formulations selected were tested to evaluate their ability to

deliver the drug into the human skin in comparison to a simple aqueous atenolol solution containing the same amount of drug (1% w/v). All the atenolol formulations markedly ($p < 0.001$) improved the amount of drug that penetrated through the skin layers compared to the simple aqueous solution. A minimum of 700% increase atenolol penetration through cat skin in the case of Formulation 3, to 750% for Formulation 5 and up to a maximum of 900%, in the case of Formulation 6 was observed. Pharmacokinetic studies and pharmacodynamic studies were performed after a clinical trial on 8 cats and the results showed the feasibility of the optimized transdermal formulation. Six of seven cats had therapeutic atenolol serum concentrations after topical administration. The topical administration of atenolol produced therapeutic atenolol levels in cats about 81% of the time. The pharmacokinetic model predicted the serum concentrations using pharmacokinetic parameters from the literature and diffusion fluxes of the study. All cats reached therapeutic concentrations of atenolol for nearly the entire time. Four cats had a single data point where the serum concentration was not in the therapeutic range (4 data points out of 21 total data points). Atenolol stability in the designed formulation was studied up to 6 months. There was no significant change in the atenolol content in the formulation. The stability studies indicate minor loss in the atenolol concentration in the designed formulation both in room temperature and at 37.5 °C. The pH is a critical component of the formulation's stability, and pharmacists should measure pH (target pH of 7) prior to dispensing this compounded medication.

Transdermal Delivery of Atenolol to Cats

by
Sumeia M. Mohamed.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Sumeia M. Mohamed, Author

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TRANSDERMAL DELIVERY OF ATENOLOL TO CATS

INTRODUCTION

Atenolol is a beta one receptor antagonist commonly prescribed to cats affected with hypertrophic obstructive cardiomyopathy. The oral tablets are highly bioavailable. However, results from several studies indicate poor therapeutic compliance by owners treating their cats during long-term oral therapy (Rush JE et al., 2008) due to difficulties in administering oral medications to feline patients. Transdermal application of atenolol is appealing since it is non-invasive and avoids both hepatic first pass metabolism and gastrointestinal degradation. Furthermore, transdermal application may also provide sustained release of atenolol and potentially improve patient owner interaction.

Chapter 1 of this thesis describes development and formulation optimization for transdermal administration of atenolol. Atenolol formulation was optimized using different absorption enhancers and co-solvents. Synthetic membrane, cloned human epidermis, and cat skin were used as permeation barriers to simulate the in vivo transdermal process. The diffusion profiles of different proposed atenolol formulas were evaluated in order to select the optimized formulation that would be used for the in-vivo part of this study. Permeation studies of atenolol were conducted in this study included three different

barriers, synthetic membrane (Nitrocellulose) with pore size of 0.45 u, cloned human epidermis and freshly collected cat skin that were mounted on Franz diffusion cells to test topical formulations ability to promote atenolol penetration through the membranes.

Chapter 2 evaluated the drug release patterns to predict the mechanism of drug release from the topical atenolol formulations. Different models for drug release were analyzed in order to determine the best fit for drug release. Zero order release, first order release, Higuchi, Korsmeyer-Peppas and Croswell models were studied as attempts to find the best fit for atenolol release pattern. A biphasic zero-order release pattern of atenolol from the topical formulation was observed. An initial zero-order burst release was followed by a slower zero-order release pattern best described the drug release from the topical test formulations.

Chapter 3 presents comparisons that were conducted to compare the different designed formulations to determine which atenolol topical formulation delivered greater drug through the membrane barriers. According to recommended tests for the comparative studies, similarity factors f_2 and difference factors f_1 were calculated for all test formulations compared to the apparent best formulation. Also partial AUC and partial AUMC were calculated to determine the best formulation that could be considered the optimized formulation for topical atenolol administration to cats.

Chapter 4 describes in-vivo testing of the previously optimized formulation in healthy cats. Topical administration of atenolol provided therapeutic concentrations (greater than 200 µg/ml) in 6 of 7 cats. Topical atenolol can be effective alternative to oral administration.

Chapter 5 describes the stability studies that were conducted during the different phases of this study. The first stability study was conducted during the in vitro phase and was aimed to determine the shelf life. The second stability study was conducted during the in vivo

studies and was an important finding for this study and helped to determine the best stability storage conditions for the compounded atenolol formulation. Storage of atenolol in light and at elevated temperature yielded minimal change in drug concentration but raising pH above 7.5 produced rapid decline in atenolol in the optimal formulation. Slightly acidic conditions also did not change atenolol concentration in the optimal topical formulation.

CHAPTER 1

DEVELOPMENT AND INVITRO STUDIES OF ATENOLOL TOPICAL FORMULATION.

Sumeia M. Mohamed

ABSTRACT

A number of different formulations were designed and tested to assess transdermal delivery of atenolol in cats. Atenolol diffusion through synthetic membrane, cloned human epidermis, cat ear and neck skin were performed utilizing a Franz diffusion cell apparatus. Transdermal drug diffusion enhancers' ethanol, glycerol, propylene glycol, polysorbate 80 and Dimethyl isosorbide (DMI) were added to the topical formulations tested for their ability to enhance drug permeation through the test membranes. DMI was either added or not to the topical atenolol formulations containing other penetration enhancers. Topical formulation with penetration enhancers showed a rapid burst of atenolol diffusion for the first two hours (35.5 to 40 ug/ml) followed by a zero-order sustained diffusion of atenolol for up to twenty-four hours after application to test membranes. This increased atenolol flux through different test membranes was greatest for synthetic membrane. The topical application of the optimized atenolol formulation to cat skin containing permeation enhancers guided the development of a transdermal atenolol drug delivery system to treat cats with hypertrophic obstructive cardiomyopathy. The optimized transdermal formulation enabled good drug delivery making the anticipation of its feasibility for transdermal application in a clinical trial in cats. The optimal topical formulation demonstrated two fluxes, the burst flux (15.7μg/cm^2/h) and a sustained flux (2.7μg/cm^2/h).

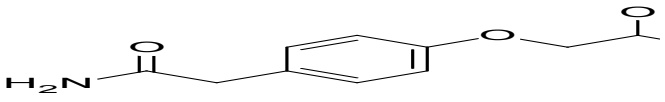
INTRODUCTION

Atenolol is one of several beta one receptor antagonists. It is commonly prescribed in cats affected with hypertrophic obstructive cardiomyopathy. The theoretical benefits of effective beta one blockade include decreased myocardial oxygen demand, reduced or abolished left ventricular outflow tract obstruction and heart rate reduction with increased diastolic filling time (Spirito P et al., 1997). In veterinary medicine atenolol is used to treat dogs and cats with cardiac problems such as an abnormal or irregular rhythm. In addition, atenolol is used to treat animals with high\blood pressure. Beta blockers are commonly used for similar purposes in human medicine. Beta blockers work by reducing the amount of oxygen that the heart muscle needs to function. Beta blockers effectively provide increased help of heart function (Antman EM, 2002).

Cats with cardiac arrhythmia are treated with atenolol in order to adjust the heart rate and reduce blood pressure. In veterinarian medicine and especially in cats; atenolol tablets are used for treatment of hypertrophic cardiomyopathy (HCM), left ventricular outflow tract obstruction and hypertension secondary to hyperthyroidism (Spirito P et al., 1997). It is superior to diltiazem as it has shown a greater ability to reduce the heart rate and consequently reduce left ventricular obstruction. Additionally, atenolol's safety profile makes it the most preferable drug among the other drugs in the same group.

The direct specific cause of how cats develop hypertrophic cardiomyopathy (HCM) is unknown (Freeman L et al., 2017); but the presence of some factors makes the disease more likely to happen. One factor implicated in the cause of Feline HCM is a dietary deficiency of taurine, an essential amino acid. Many cat foods lack this required supplement. The association of the disease with taurine deficiency caused the addition of taurine to cat's food to make sure they have a sufficient amount. Also tuna or fish based food are more likely to facilitate hyperthyroidism which leads to overload on the cat's heart, thickening of the heart muscle and developing an enlarged heart in cats (Beth H, 2016). Other chemicals in canned food can be factors that might contribute to heart disease. Lack of antioxidants can also be a leading factor for cardiomyopathy (Beth H, 2016).

There is an association between the modern home environment and cats developing heart disease. In some feline families there is a genetic predisposition for this condition (cardiomyopathy) like Maine coon cats. But generally, the association cannot be proven, although the disease has been documented with a higher incidence rate in American short hair and Persian cats. The disease most commonly occurs in cats between ages of 5 to 7 years, but recorded cases have ranged from 3 months to 17 years. The incidence rate of this disease is higher in males (Rush J et al., 2002).



Atenolol (free base) is isopropyl-amino-propanol derivative. It has a molecular weight of 266. It is a relatively polar hydrophilic compound with a water solubility of 26.5 mg/mL at 37°C and a log partition coefficient (octanol/water) of 0.23. It is freely soluble in 1N HCl (300 mg/mL at 25°C) and less soluble in chloroform (3 mg/mL at 25°C).

In veterinary medicine, atenolol like many other drugs, is not FDA approved for use in animals and is not available from a veterinary pharmaceutical manufacturer. Instead, veterinarian's use atenolol drug products prepared by a compounding pharmacy or FDA approved tablets for human use. Cats with heart disease receive an atenolol dose of 6.25 to 12.5 mg once or twice a day, usually tablets or pills.

Administration of oral tablets to cats can be a challenging issue for owners leading to difficulty in maintaining therapeutic compliance in cats that is not always attainable (Siven M et al., 2017). However not all cats respond in the same manner. Therefore, there is an increasing demand to develop an alternative route of administration for atenolol to cats. In human, billions of transdermal drug dosage forms for several drugs are produced every

year. Thus, clinical experience suggests use of transdermal drug formulations to administer atenolol for cats may be beneficial.

Atenolol is commonly prescribed in cats affected with hypertrophic obstructive cardiomyopathy. The oral tablets are highly bioavailable. However, results from several studies indicate poor therapeutic compliance by owners to treat their cats during long-term oral therapy (Rush JE et al., 2008) due to difficulties in administering oral medications to feline patients i.e. cat scratches, bites or owner laziness. Transdermal application of atenolol is appealing since it is non-invasive and avoids both hepatic first pass metabolism and gastrointestinal degradation. Furthermore, transdermal application may also provide sustained release of atenolol and potentially improved patient owner interaction.

A huge help to cat owners over the past several years has been attained by transdermal drug delivery. With growing numbers of cats developing chronic diseases like kidney dysfunction, cardiac disease, diabetes, urinary disease and hyperthyroidism means medication can literally be lifesaving. However, administration of medicine to cats is not easy especially in long term therapy. Thus, transdermal medications can absolutely be an alternative for drug administration and ultimately save cat lives. There are a number of drugs that are administered to cats via transdermal route

Transdermal medications which have been made available for cats include methimazole (Tapazole) (to treat hyperthyroidism), benazepril, prednisolone (Omnipred), fluoxetine (Prozac), and Amlodiipine (Norvasc) are used for blood pressure management. Also

mirtazapine (Remeron) to stimulate appetite, an antibiotic named enrofloxacin. Amitriptyline is to control behavioral issues and as an analgesic (https://ggvcp.pharmacy/2018/04/26/ggvcps-new-transdermal-pen-now-available).

Unfortunately, not every available drug can be a substrate for transdermal formulation.

One study conducted on 13 cats diagnosed with hyperthyroidism, treatment with a transdermal methimazole formulation was applied on the internal ear pinna at a dose of 5 mg. This prospective clinical study suggested that transdermal methimazole is an effective and safe alternative to conventional oral formulation (Lecuyer M et al., 2006). Another study found similar results that transdermal application of methimazole used as alternative dosage form for therapy in cats was efficacious (Sartor LL et al., 2004).

Administration of atenolol has not proven to change the outcome in affected cats. However, many cardiologists prescribe atenolol in the setting of dynamic left ventricular outflow tract obstruction using highly bioavailable oral tablets (Schober KE et al., 2013).

The veterinary literature provides few publications on atenolol administration to cats. One study evaluated the pharmacokinetics and pharmacodynamics variables of a single atenolol dose given intravenously and orally to cats (MacGregor JM et al., 2008). Post administration at 6 and 12 hours they reported significant decreases in heart rate; following isoproterenol challenge in all cats with a minimum atenolol plasma concentration of more than 260ng/ml. Another small study compared atenolol pharmacodynamics after oral and transdermal administration in healthy male cats. This group found cats receiving oral atenolol reached therapeutic concentrations after oral administration, whereas cats

receiving the transdermal formulation had atenolol concentrations below 260 ng/ml at the same time point (Macgregor JM et al., 2008). These results raise questions specifically regarding the direct substitution of transdermal doses at equivalent oral doses, and even possible variability of compounded medications.

This project seeks to develop a transdermal formulation that will facilitate diffusion of atenolol across cat skin when a topical formulation is being used as the route of drug administration. Skin is by far the largest organ in the body, covering a large surface area and receiving about one third of the entire blood circulation. The main role for skin is to exert a protective function in terms of preventing external microbes and chemicals penetrating into the body. It also plays a role in temperature control and provides protection for the body against mechanical shocks and injury. However, the very large surface area of the skin means it is potentially attractive in application for topical drug delivery. The transdermal drug delivery approach has many advantages over the more conventional oral treatment. These include the avoidance of first pass metabolism, treatment can be quickly started or stopped, the rate of systemic delivery can be more uniformly maintained and sometimes provide a sustained release of the drug and potentially better patient compliance is achieved. Obviously, the transdermal route provides the clinician with a possible extra therapeutic option for patient therapy. Disadvantages include the potential for localized irritation, development of allergies and delayed drug action due to difficulties associated with the time necessary for the drug to diffuse through the skin (Blank HI et al., 1969).

Therefore, the aim of this study is to address the problem of the formulation of atenolol for transdermal delivery by optimizing the concentration of drug within the carrier gel. Secondly this study aims to assess serum atenolol concentration and therapeutic response as it relates to dose and plasma concentration.

MATERIALS AND METHODS

Atenolol 100 mg tablets were obtained from TEVA (Gardna CA), Ethanol USP Grade from Pharmaco, AAPER from Brookfield. C. T. New York, 10013. Dimethyl isosorbide (DMI) from CRODA (NJ, USA 300 Columbus Cir # A, Edison, NJ 08837). Glycerol from EMD chemicals Inc (110 EMD Blvd, Port Wentworth, GA 31407 (USA), Propylene glycol and Triethanolamine from J.T. Baker Inc (600 Broad St, Phillipsburg, NJ 08865 USA). Polysorbate 80 obtained from Acumedia (620 Lesher Pl, Lansing, MI 48912. Houston Texas, USA) and Carbomer 934 obtained from Spectrum (V Gardena, CA 1434 W Gardena Blvd, Gardena, CA 90247).

Membranes filters used were transfer medium pure nitrocellulose (0.45 um) Trans–Blot obtained from BIO-RAD, (4000 Alfred Nobel Dr, Hercules, CA 94547, USA). Cloned human epidermis was obtained from MatTek Corporation (200 Homer Ave, Ashland, MA 01721). Fresh cat skin obtained from Oregon State University, College of Veterinary Medicine, Cardiology department, Necropsy Laboratory.

Preparation of atenolol formulations

Optimization of topical atenolol formulation: different formulations were produced by changing the percentage of the ingredients. The next step was testing atenolol diffusion through various membranes to select the optimal formula for the in vivo study phase. Different atenolol topical formulations are shown in Table (1) containing different percentages of ingredients that were prepared and subjected to in vitro permeation of atenolol across synthetic membrane, cloned human epidermis and freshly prepared cat skin.

The selection of the gel ingredients

The gel contains propylene glycol and glycerin as humectants, co-solvents and pharmaceutical excipients. A surfactant (Polysorbate 80) was added to aid in emulsification and to improve drug transportation. Ethanol also was added to improve the skin permeation of atenolol. Dimethyl isosorbide (DMI) was used as a drug carrier and transdermal enhancer. The gel base was either 1% or 0.75% carbomer that was neutralized with 0.45% tri-ethanolamine to produce a pH of 7.

Atenolol formulations were prepared using different concentrations of different enhancers (Table 1.1).

Formulation code	Percent Atenolol	Percent Tween 80	Percent Glycerol	Percent Propylene glycol	Percent Ethanol	Percent DMI
F1	1	5	5	15	5	0
F2	1	5	10	15	5	0
F3	1	5	15	15	10	0
F4	1	5	15	15	10	5
F5	1	5	15	15	10	10
F6	1	5	15	15	10	15
F7	1	2.5	15	15	5	5
F8	1	2.5	15	15	10	10
F9	1	2.5	15	15	10	15

Table 1.1: Composition of various Atenolol formulations (mg). All formulations contain 0.45 % triethanolamine and either 1% carbomer (F7, F8, F9) or 0.75% carbomer (F1, F2, F3, F4, F5, F6).

Gel formulation

Fifty grams of a clear (1% atenolol gel) was prepared as follows. The gel was formulated by adding glycerin to the propylene glycol and then mixing with water to make a dispersion. Then atenolol was added to this solution, followed by the surfactant (Polysorbate 80) and DMI. Carbomer was dispersed in water in another beaker along with the triethanolamine. The two mixtures were mixed together and triturated thoroughly to form a gel. The gel was stored at room temperature after preparation (22 to 25 °C). Figure 2.1 show the process of gel formation. Table 2.2 shows the Standard operating Procedure (SOP) developed to make the atenolol gels for the study.

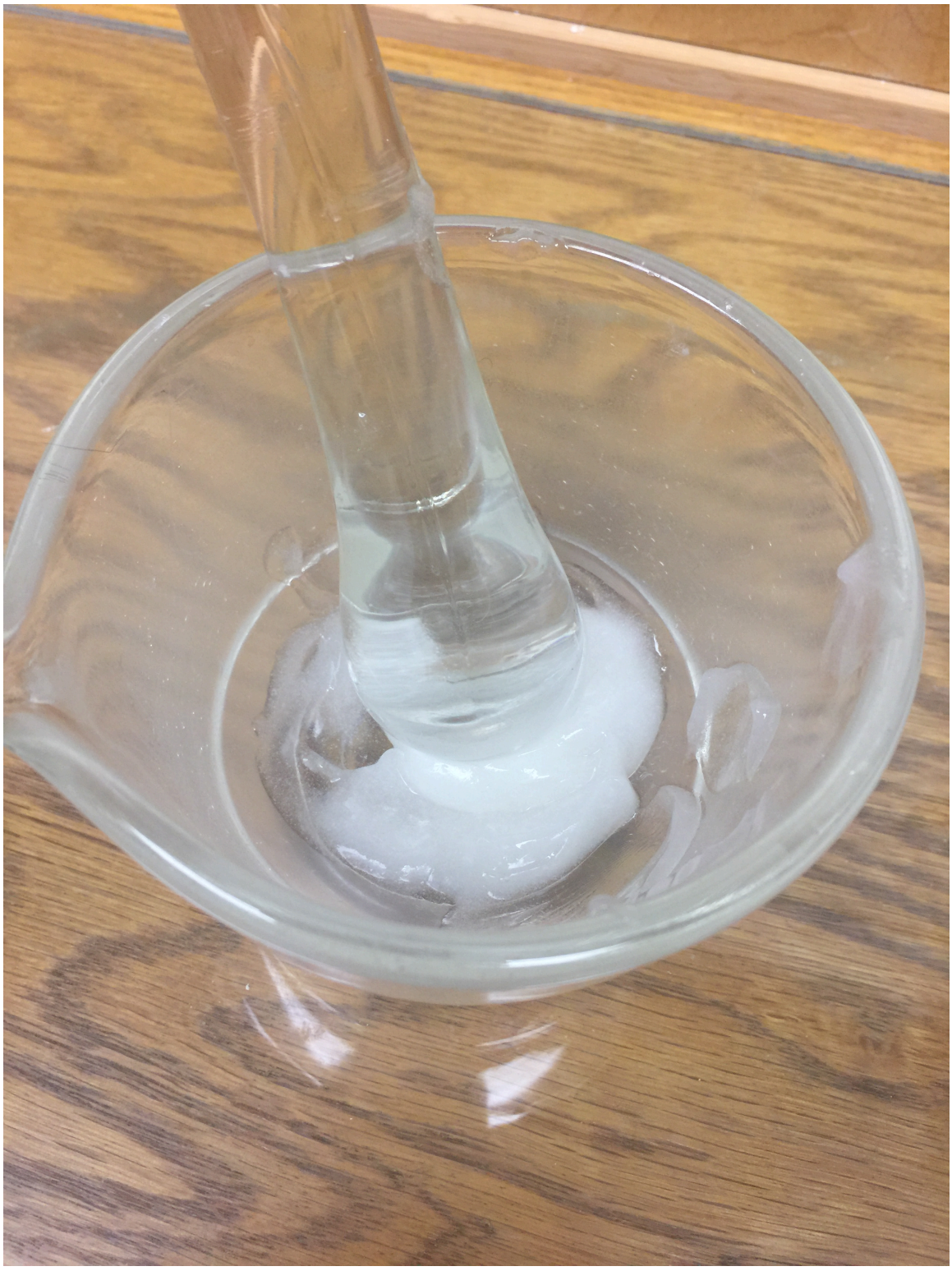


Figure 1.1 The appearance that all topical gel formations had.

Table 1.2. Standard Operating Procedure (SOP) for making Transdermal Gels

Ingredient	Amount
Atenolol	12.5g/ 100 ml
Propylene glycol	15g/ 100ml
Glycerin	15g/ 100ml
Polysorbate 80 (Tween 80)	15g/ 100ml
Dimethylisosorbide (DMI)	15g/ 100ml
Alcohol USP (95% Ethanol)	10g/ 100ml
Carbopol 934 (Carbomer)	0.75g/100ml
Triethanolamine (Trolamine)	0.45g/100ml
Purified Water USP	qs (~16.3ml)

This will generate 100 (1 mL) syringes each having 12.5 mg for each 0.1 ml (125mg/ml). Scale down the amounts to produce fewer syringes.

1. Using 100 mg tablets of atenolol, count out sufficient number of tablets for the amount needed. For 100 ml, 125 tablets will be needed. Keep separate from the gel. Weigh_____
2. Weigh into a separate beaker 15 grams of propylene glycol for the final 100 ml. Weigh _____
3. Weigh out 15 grams of glycerin and add to the propylene glycol yielding a propylene glycol/glycerin mixture. Weigh_____
4. Weigh out 15 grams of polysorbate 80 and add to the propylene glycol/ glycerin mixture. Weigh _____

5. Weigh out 15 grams of Dimethylisosorbide (DMI is a strong solvent and carrier) and add it to the propylene glycol/glycerin /polysorbate 80 mixture. Weigh_____
6. Weigh out 10 grams of alcohol USP and add it to the propylene glycol/glycerin/polysorbate 80 mixture. Weigh_____
7. To the propylene glycol/glycerin/polysorbate 80/DMI mixture, add 6.3 grams of purified Water USP. Weigh_____
8. Weigh out 0.75 grams of carbopol 934 in a separate weigh boat.
   Weigh _____
9. Weigh out 10 grams of purified water USP in a separate beaker and add the carbopol p34 and triturate well mixing the carbopol thoroughly into the water.
   Weigh _____
10. Add the carbopol water mixture to the propylene glycol/glycerin/polysorbate 80/DMI mixture and continue mixing until the carbopol is thoroughly incorporated into the mixture.
11. Weigh out 0.35 grams of triethanolamine (Trolamine) and add to carbopol/propylene glycol/glycerin /polysorbate 80/DMI mixture and add gradually Trolamine while mixing until gel is formed while monitoring the pH (keep the pH at 7.0).
    Weigh _____
12. To the carbopol/Trolamine/propylene glycol/glycerin/polysorbate 80/DMI mixture add the atenolol tablets.

13. Thoroughly grind the tablets into an extremely fine powder and mix into the gel mixture.
14. Measure the pH of the gel as best results occur for topical application if pH is 7.0. pH above 7.4 may cause the gel to degrade and discolor. Store at 22-25 ^{o}C and protected from light.

Instrumentation

A simple and effective High Performance Liquid Chromatography method was developed and validated to determine atenolol concentrations in various types of samples including cat's plasma. Atenolol was analyzed using HPLC-UV detection. Chromatographic analysis was carried out using a Shimadzu Prominence High performance liquid chromatographic system (Kyoto, Japan) LC-2010A HT model and equipped with a dual UV detector and pump.

All reagents and solvents were HPLC grade. Methanol and acetonitrile were purchased from Fisher Scientific (755 US-202, Branchburg, NJ 08876; USA). Glacial acetic acid which is used to produce a pH of 3 in the mobile phase was obtained from VWR international Philadelphia (PA 2039 Center Square Rd. USA).

The HPLC analysis was performed using a 4.6x150 mm column (Kinetex, Phenomenex (411 Madrid Ave, Torrance, CA 90501 CA, USA) packed with 5-um C-18 chromatographic medium and connected to a pre-column (security guard 2.1x4.6 mm, Kinetex, Phenomenex, CA, USA). The flow rate was 1 ml per minute and the detector

wave length was set at 230 nm with the column temperature fixed at 25 °C. The injection volume was 20 µl, and the run time was 5 minutes (Kumar N et al., 2010)

The first part of this study is the creation of a HPLC atenolol calibration curve. Serial dilutions of 1mg/ml stock solution of atenolol was performed to prepare different concentrations starting from 40, 20, 10, 5, 2.5, 1, 0.75, 0.5, to 0.1 (µg/ml) to run through the HPLC (Shimadzu). The mobile phase was an acidic pH 3 produced by the addition of few drops of glacial acetic acid. The mobile phase consists of 60:20:20 acidic water, acetonitrile and methanol and sonicated for one hour before use. The flow rate is 1ml/minute. The corresponding AUC of atenolol HPLC peaks produced by the prepared atenolol standard solutions are plotted versus the atenolol standard concentrations that produced them and a calibration curve was prepared and validated by three sequential HPLC runs that produced equivalent standard calibration curves. Validation of the HPLC assay method was performed according to FDA recommendations for assessment. The assay of the HPLC methods includes assay's linearity, LOD, LOQ (Spankis M et al., 2010).

Linearity

Upon plotting the chromatograms average ratio of peak areas (AUC) of Atenolol versus concentration range (0.1-40 µg/ml) a straight line was produced (Figure 1.2). Linear regression on the calibration curve was applied. ANOVA test (One way) was used to obtain the equation ($y = 165261x + 48470$) and $R^2 = 0.9989$. The relationship in the method was clearly linear based on the high R^2 (Correlation coefficient). The lower limit that were detected (LOD) was 0.045 µg/ml while the limit of quantification (LOQ) of atenolol was 0.1 µg/ml. linear relationship between the peak ratios and atenolol concentrations in the range of 0.1 to 40 ug/ml with $R^2 = 0.9989$.

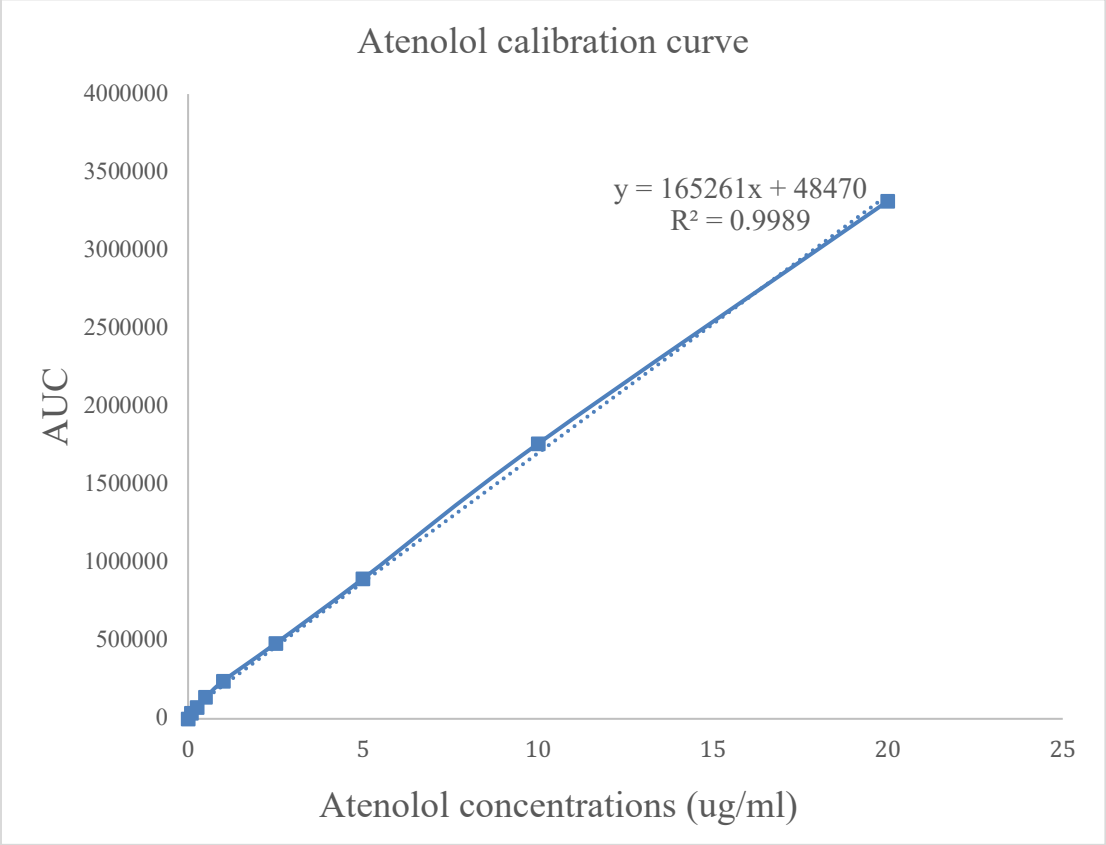


Figure (1.2 a) Validated Standard calibration curve of atenolol.

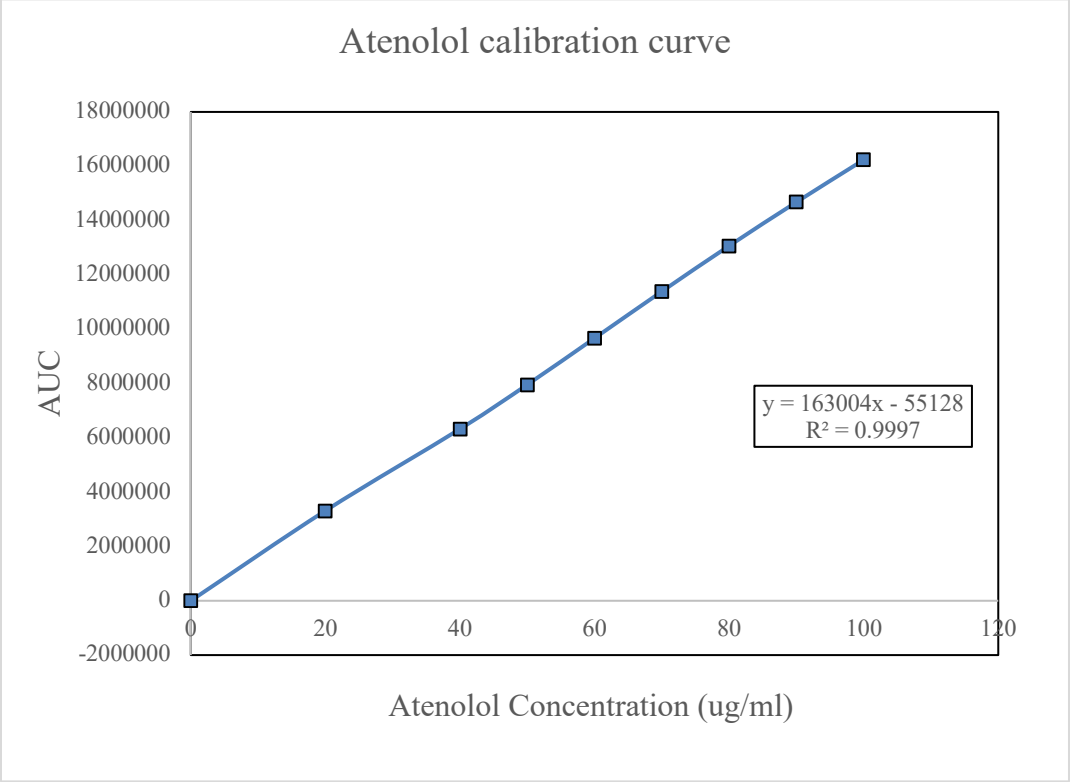


Figure (1.2 b) Validated Standard calibration curve of atenolol at higher concentrations, concentration range of (20 to 100 ug/ml).

In vitro skin permeation study

Atenolol Permeation studies through synthetic membrane, cloned human Epidermis and cat skin were conducted using Franz cells; PermeGear vertical glass diffusion cells from HAAK-L (Figure 1.3). Six Franz diffusion cells (Hanson Research Chatsworth CA USA), with a surface area of 6.43 cm^2 and a receptor cell volume of 14 ml were used. The receptor solution was a phosphate buffer solution (PH 7.4) stirred at 600 rpm and maintained at 37^oC by use of a heated circulating water heater with pump delivering heated water to each chamber. The membrane or skin initially was allowed to hydrate in the Franz Cells for an hour. During this time the cells were occasionally turned upside down in order to allow the escape of any air bubbles that might develop on underside of the membrane or skin. Then, each membrane or cat's skin was treated with one gram of the selected test formulation. A control experiment was also run in which the membrane or skin was treated by a one percent solution of atenolol.

Figure (1.3) Waake Franz upright diffusion cells set system used in testing atenolol formulations.

Sampling

Drug permeation through test membranes was allowed to continue for 24 hours. At chosen times, a 100 µl volume of solution was withdrawn from each Franz cell receiver solution and replaced with the same volume of phosphate buffer solution to compensate for volume loss. The samples withdrawn were analyzed by direct injection into the HPLC. Atenolol concentrations were determined from the prepared calibration curve of standard atenolol solutions. In all cases, atenolol concentration values obtained for each aliquot was corrected for the progressive dilutions developing during the course of the Franz diffusion cell experiment (Khan et al., 2005).

Pilot study

A study was conducted to determine the best sampling intervals. Initially, sampling was done every 15 minutes through 24 hours depending on the amount of diffused drug into the Franz diffusion cell receptor compartment. Different membranes and skin tissues were used to determine atenolol permeation from test formulation. Membranes and Skin Tissues that were used are the following:

1- Synthetic membrane

An inert synthetic membrane made from cellulose acetate was used for the initial permeation studies for Atenolol to evaluate promising test formulations. The synthetic membrane possessed a pore size of 0.45um.

2- Cloned human epidermis

Cloned human epidermis was obtained from MAT-TEK Inc. Comparison of diffusion of Atenolol through cloned human epidermis to synthetic membranes was performed to confirm the synthetic membranes results.

3- Cat skin

Freshly collected cat's skin by faculty of the College of Veterinary Medicine, Cardiology Department during necropsy were utilized to assure the optimal test formula would provide transdermal absorption of atenolol through cat skin in the *in-vivo* study.

Using necropsy samples with permission of the Oregon State University, College of Veterinary Medicine's Institutional Animal Care and Use Committee, selected atenolol formulations were applied to both the pinna and nape of cats. These two sites are specifically targeted as areas difficult to remove/ingest drug despite the feline species' tendencies to be fastidious groomers. After removal of epidermal hair, the skin was cleansed and any adhering subcutaneous tissue and blood vessels were removed. Franz-Chin diffusion cells were used to measure atenolol permeability through the excised skin placed between the half cells.

The test membranes were mounted between the donor and receptor compartments. The effective diffusion area of the cell was 6.43 cm^2 and with a receptor volume of 14 ml. The receptor medium was phosphate saline buffer pH 7.4. The prepared atenolol gel was placed on one side (upper side) of the membrane. The receptor compartment was bounded by a water jacket to retain the temperature at 37°C. The receptor fluid was stirred by a Teflon-

coated magnetic stirrer (Jug et al., 2009). At specific time intervals, samples in the acceptor chamber were collected at times 0, 2, 4, 6, 8, 12, 24 hours and replaced with an equal volume of receptor media for drug content determination. Drug concentration was determined using HPLC (Shimadzu).

RESULTS

In vitro drug release for all formulations of the atenolol gels

The diffusion of atenolol through synthetic membrane from formulations 1 to 6 is presented in Figure 1.4. and tables 1.3 to 1.8. Formulation 3 showed rapid release and diffusion of atenolol through the cellulose nitrate membrane. Almost 90 ug/ml was measured in the receptor solution after six hours and 106.7 ug/ml at twenty fours. Formulation 6 in comparison released less atenolol at 6 hours and 24 hours (48.75 ug and 104.1 ug/ml respectively).

Atenolol permeation through the cloned human epidermis was much less than that of synthetic membrane and cat skin as shown in Figure 1.5 and table 1.9. Atenolol concentrations in the receptor compartment after 8 hours was 5.05 ug/ml $\pm$ 2.18, 5.878 ug/ml $\pm$ 3.86 and 16.26 ug/ml $\pm$ 5.84 for formulations 3, 5, and 6 respectively.

In the Figure 1.6 and table 1.10 atenolol diffusion through freshly obtained cat skin, permeation studies for the three selected formulations showed that formulation 6 with 15% DMI had the highest drug permeation over 24 hours (76.56 ug/ml $\pm$ 20.44) followed by formulation 5 at 56.1 ug/ml $\pm$ 10.01 (with 10% DMI) and lastly formulation 3 at 45.61 ug/ml $\pm$ 9.26 with 0% DMI.

The concentration versus time diffusion of atenolol through Synthetic membrane, cloned human skin, and cat skin are presented in tables 1.3-1.10. Table 1.11 present the statistical testing of the fluxes for the different diffusion fluxes through cat's skin. Due the large variance no statistical differences were observed in the fluxes (burst or sustained phases) for formulations 3, 5, and 6.

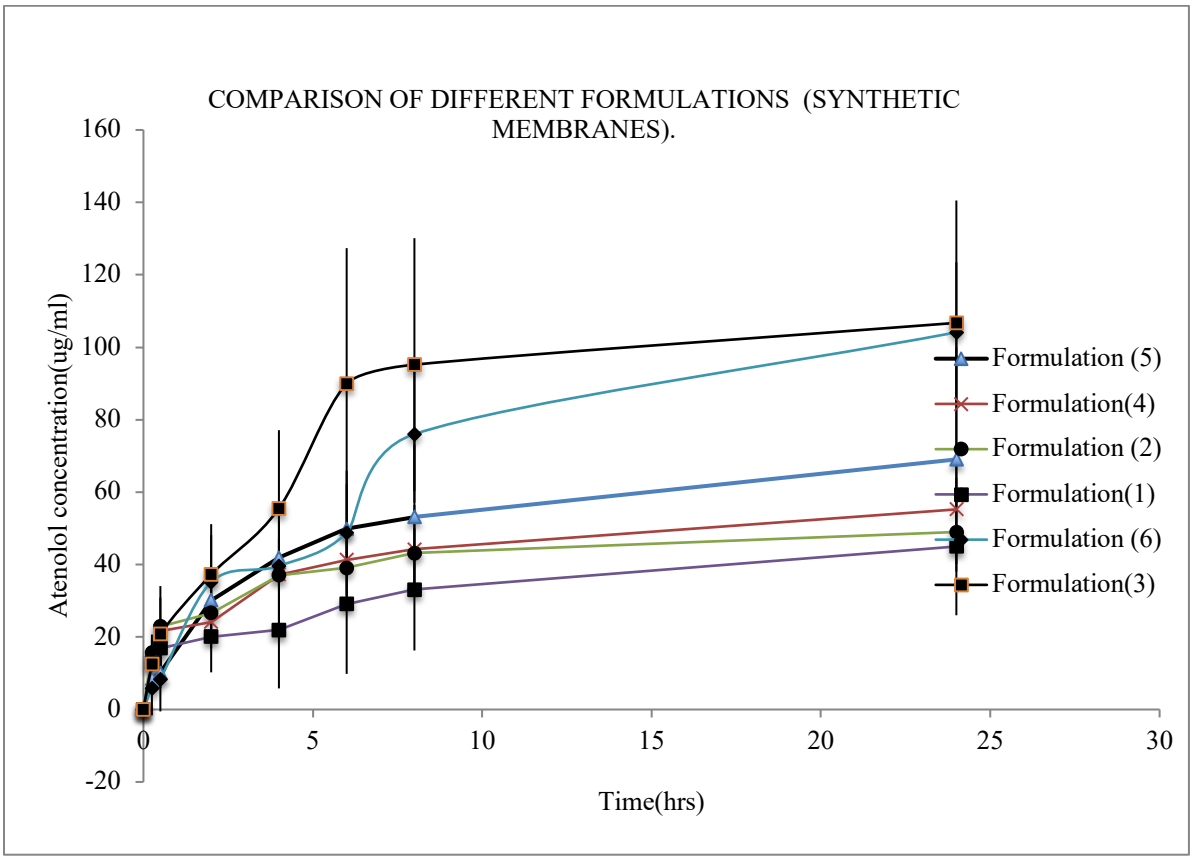


Figure 1.4 Atenolol test formulations 1-6 diffusion through synthetic membrane.

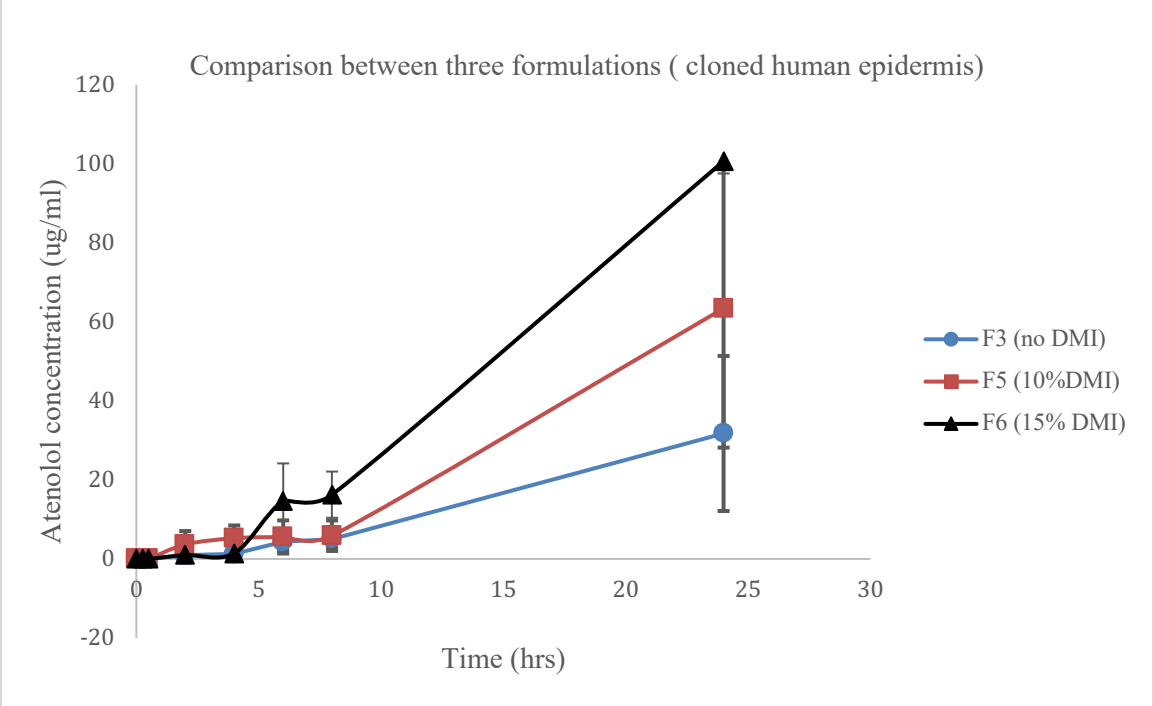


Figure 1.5 Permeation of the best three gel formulations (3, 5, 6) of atenolol through cloned human epidermis.

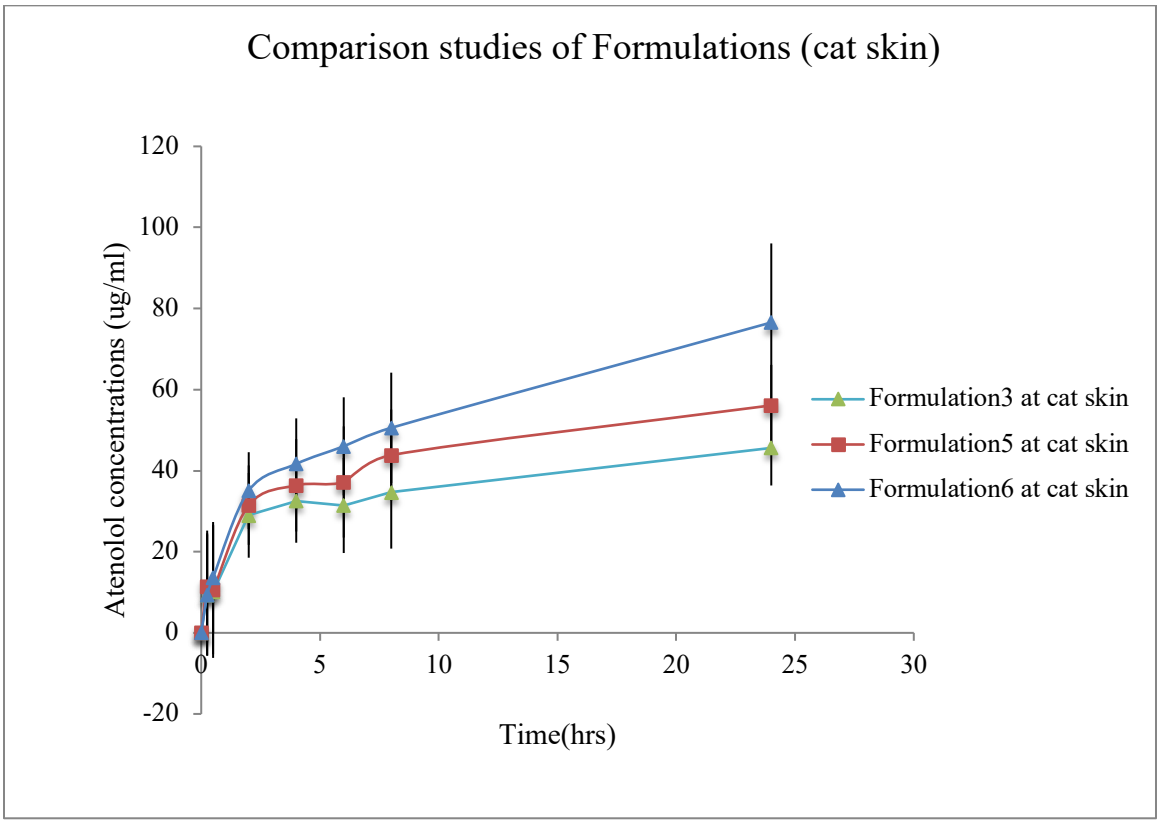


Figure 1.6 Permeation of atenolol from formulations 3, 5, and 6 through excised cat skin.

Tables 1.4 to 1.9 along with Figure 1.4 present the mean concentration time curves of atenolol measured in the Franz diffusion receptor cells for test formulations 1-6 diffusion through synthetic membrane. The mean values of diffusion of atenolol through cloned human epidermis is reported in Table 1.10 and Figure 1.5 while Table 1.11 and figure 1.6 presents the atenolol diffusion from formulation 3, 5, and 6 through cat skin.

Atenolol flux through the membranes appears to have two different phases (Tables 1.11and 1.12). The first flux is a Burst Flux which gave the following values: 7.67 to 56.73 ($ug/cm^2/h$) for Formulation 3. For Formulation 5 the Burst Flux (J_1) ranged from 13.24 to 74.93 ($ug/cm^2/h$) and from 16.516 to 75.07 ($ug/cm^2/h$) for Formulation 6.

The second flux is more consistent and shows a sustained release of atenolol as shown in Tables 1.14 and 1.15. The second (sustained) Flux J_2 ranged from 0.97 to 1.98 ($ug/cm^2/h$) for Formulation 3. The sustained flux for formulation 5 ranged from 1.19 to 2.48 ($ug/cm^2/h$) while that for Formulation 6 has the following range 1.07 to 3.5 ($ug/cm^2/h$).

Table 1.13 shows the t-test for F3, F5 and F6 initial (Burst) Fluxes and as shown in this table, F3 and F5 are statistically different. t-test for Formulation 3 to Formulation 6 was statistically different while Formulation 5 and 6 were not statistically different.

Table 1.15 compares the second (sustained) fluxes for Formulation 3, 5 and 6. It shows that F3 is statistically different to F5. According to t -test, F5 is not statistically different to F6.

The solubility of atenolol in water is 26.5 mg/ml. The solubility of atenolol in formulation 6 was slightly greater than 100 mg/ml. The solubility of atenolol was determined to be

slightly greater than 100 mg/ml that is 4 times greater in solubility in the co-solvent system of Formulation 6 than water. The increase in atenolol solubility in the co-solvent does not fully account for the increase in permeation of atenolol through cat skin revealing that the use of penetration enhancers is needed to produce sufficient atenolol penetration.

Formulation 1 (synthetic membrane)	Atenolol average conc. (ug/ml)	Std
Time (hrs)	0	0
0	12.879	13.986352
0.25	16.95	13.605464
0.5	20.15	8.888546
2	21.972	12.546629
4	29.082	11.334588
6	33.134	15.345823
8	40.009	17.239146
24	45.879	23.54003

Table 1.3 Atenolol concentration versus time for formulation 1 (synthetic membrane)

Formulation 2 (synthetic membrane)	Atenolol conc.	Std
Time(hours)	(ug/ml)	13.09456
0	0	13.22345
0.25	15.809	15.67409
0.5	22.915	18.79430
2	26.645	12.87601
4	37.07	17.77893
6	39.099	15.44321
8	43.134	24.56093
24	49.009	25.33891

Table 1.4 Atenolol concentration versus time for formulation 2 (synthetic membrane)

Formulation 3 synthetic membrane	Atenolol conc.	Std
Time (hours)	(ug/ml)	
0	0	0
0.25	12.621	9.23409
0.5	20.845	11.3294
2	37.263	9.99739
4	55.54	14.4610
6	89.98	18.2237
8	95.2	20.3204
24	106.74	17.4590

Table 1.5 Atenolol concentration versus time for formulation 3 (synthetic membrane).

Formulation 4 (synthetic membrane)	Atenolol concentrations	Std
Time (hrs)	(ug/ml)	
0	0	016.88745
0.25	13.249	19.456321
0.5	21.725	20.345018
2	24.135	22.308754
4	37.27	25.745609
6	41.329	22.456091
8	44.24	20964309
24	55.249	21.665833

Table 1.6 Atenolol concentration versus time (formulation 4) through synthetic membrane.

Formulation 5 (synthetic membrane	Atenolol concentrations	Std
Time (hrs)	(ug/ml)	
0	0	0
0.25	8.479	15.47809
0.5	10.15	18.67402
2	30.15	16.90737
4	41.942	13.98540
6	49.88	22.45691
8	53.164	23.45309
24	69.049	24.36570

Table 1.7 Atenolol concentration versus time for formulation 5 (synthetic membrane).

Formulation 6 (synthetic membrane)	Atenolol concentrations	Std
Time (hours)	(ug/ml)	
0	0	0
0.25	5.907	17.54308
0.5	8.385	18.45992
2	35.167	20.33858
4	39.619	21.11609
6	48.75	22.50390
8	76.027	25.07693
24	104.129	25.11870

Table 1.8 Atenolol concentration versus time for formulation 6 (synthetic membrane).

Time (hours)	Formulation 3 Atenolol (ug/ml)	Std	Formulation 5 Atenolol (ug/ml)	Std	Formulation 6 Atenolol (ug/ml)	Std
0	0	0	0	0	0	0
0.25	0	0	0	0	0	0
0.5	0	0	0	0	0	0
2	0.941	0.779583	3.661	3.391709	0.957	1.432
4	1.227	0.876	5.241	3.230809	1.428	1.818679
6	4.354	2.180925	5.562	4.194008	14.637	9.534
8	5.05	2.180925	5.878	3.862449	16.263	5.842823
24	31.746	19.62411	63.419	35.2479	100.67	3.1234

Table 1.9 Atenolol concentration versus time for formulations 3, 5, and 6 through cloned human epidermis.

Time (hours)	Formulation 3 (ug/ml)	std	Formulation 5 (ug/ml)	Std	Formulation 6 (ug/ml)	Std
0	0	0	0	0	0	0
0.25	9.451	15.11	11.43	13.79	9.38	14.9044
0.5	10.167	16.34	10.7	15.96	13.65	13.6845
2	28.98	10.44	31.50	9.79	35.5	9.46876
4	32.5	10.27	36.39	11.39	41.67	11.2334
6	31.422	11.72	37.21	13.71	46.01	13.6419
8	34.68	13.89	48.83	11.24	50.57	19.4997
24	45.61	9.26	56.10	10.01	76.56	20.4356

Table 1.10 Atenolol concentration versus time for formulations 3, 5, and 6 permeation through cat skin.

Formulation code	Cat skin studies Burs Flux (J_1) (ug/cm^2/hr)	Sustained Flux (J_2) (ug/cm^2/hr)	t- test	t- test results
Formulation 3	10.618	1.504	F3 and F5	Statistically different
Formulation 5	11.528	1.942	F3 and F6	Statistically different
Formulation 6	15.723	2.734	F5 and F6	Not Statistically different

Table 1.11 Atenolol flux through cat skin.

Atenolol flux and the statistical analysis of the gel formulations were calculated and are presented in Tables 1.11 through 1.15. The initial burst flux is consistent with absorption of atenolol through cat skin after topical application. The second atenolol flux calculated also coincides with atenolol topical application. A rapid initial rate of diffusion followed by a sustained zero-order release of atenolol fit drug release and absorption through cat skin. There was no statistical differences in the fluxes between atenolol formulations.

The Burst Flux for each formulation is presented in Table 1.11 and it was found that J_1 (Burst Flux) for the average concentrations of Formulation 3 is (10.1618 μg/cm²/h), also for Formulation 5 (11.524 μg/cm²/h) and (15.723 μg/cm²/h) for Formulation 6. The t- test showed all the Formulation are statistically not different.

Franz cell no	Formulation 3 First Flux (J_1) (µg/cm²/hr)	Formulation 5 First Flux (J_1) (µg/cm²/hr)	Formulation6 First Flux (J_1) (µg/cm²/hr)
FRANZ1	27.22	62.248	66.06
FRANZ2	56.73	77	71.74
FRANZ3	7.67	58.22	73.98
FRANZ4	13.245	74.93	75.07
FRANZ5	13.326	62.31	73.659
FRANZ6	15.597	22.195	18.805
FRANZ7	14.47	15.361	16.516
FRANZ8	16.025	15.52	18.084
FRANZ9	14.838	16.57	18.26
FRANZ10	13.857	18.18	17.568
FRANZ11	12.53	13.24	16.93

Table 1.12 The initial flux (J_1) for each formulation in cat skin.

Formulation's first (Burst) flux (cat skin)	t- test
Formulation 3 & 5	2.31 (significantly different)
Formulation 3 & 6	2.46 (significantly different)
Formulation 5 &6	0.276 (not significantly different)

Table 1.13 t-test results comparing test formulations**.**

FRANZ NO	FORMULATION 3 FLUX (J2)	FORMULATION 5 FLUX(J2)	FORMULATION 6 FLUX (J2)
FRANZ1	1.07	1.362	1.64
FRANZ2	1.08	1.19	1.43
FRANZ3	0.97	1.396	1.07
FRANZ4	1.369	1.224	3.109
FRANZ5	1.515	1.5	3.78
FRANZ6	1.7	1.634	3.5012
FRANZ7	1.909	2.48	2.66
FRANZ8	1.94	2.12	2.9
FRANZ9	1.98	2.27	3.211
FRANZ10	1.89	2.31	3.405
FRANZ11	1.06	1.98	3.7

Table 1.14 The second Flux (J_2) values of the three best formulations

FORMULATION 'S SECOND (SUSTAINED) FLUX	t- test
FORMULATION 3 & 5	2.52 (statistically different)
FORMULATION 3 & 6	4.2 (statistically different)
FORMULATION 5 &6	1.2 (statistically different)

Table 1.15 t- test results comparing test formulations.

Cat skin permeability (neck versus ear)

Diffusion of atenolol through cat neck skin compared to cat ear skin is presented in Figures 1.7—1.9 for test formulations 3, 5, and 6 respectively. Atenolol diffusion through cat ear skin is slightly better for each test formulation with Formulation 6 providing greater skin penetration of atenolol with more consistent skin permeation rates. Based on the diffusion profiles for F3, F5, and F6, the ear skin produced more drug flux into the receiver compartments. Application of drug to the ear skin is more convenient for the use as site of application for cats and their owners as they don't need to shave ears as would be the case for application of drug to the neck skin.

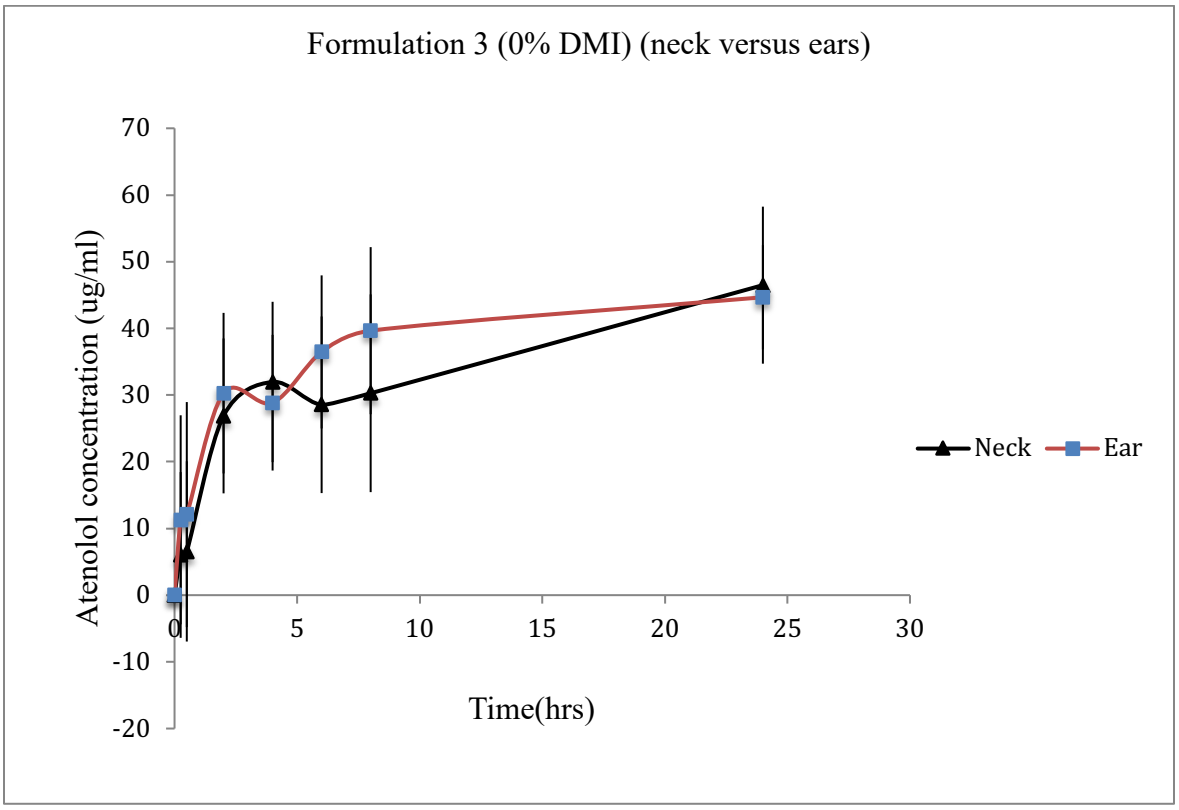


Figure 1.7 Comparison of atenolol concentration time curves for formulation 3 through cat neck skin versus cat ears skin.

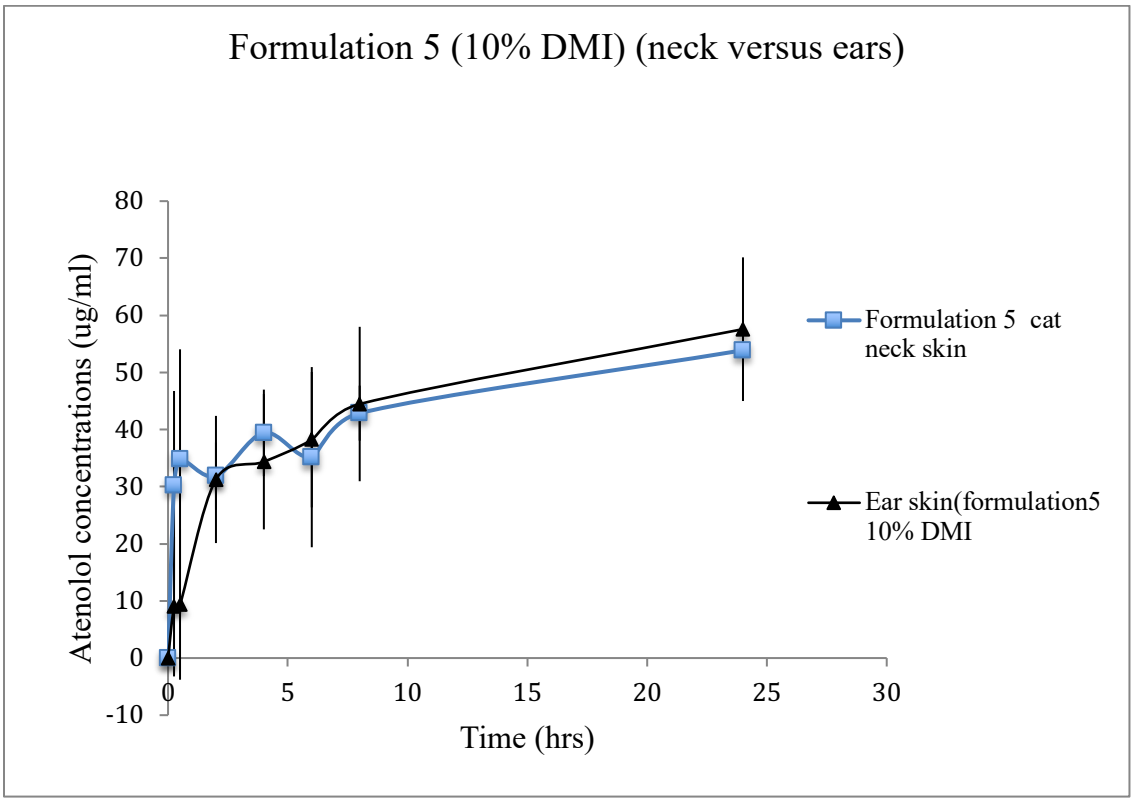


Figure 1.8 Comparison of atenolol concentration time curves for formulation 5 through cat neck skin versus cat ears skin

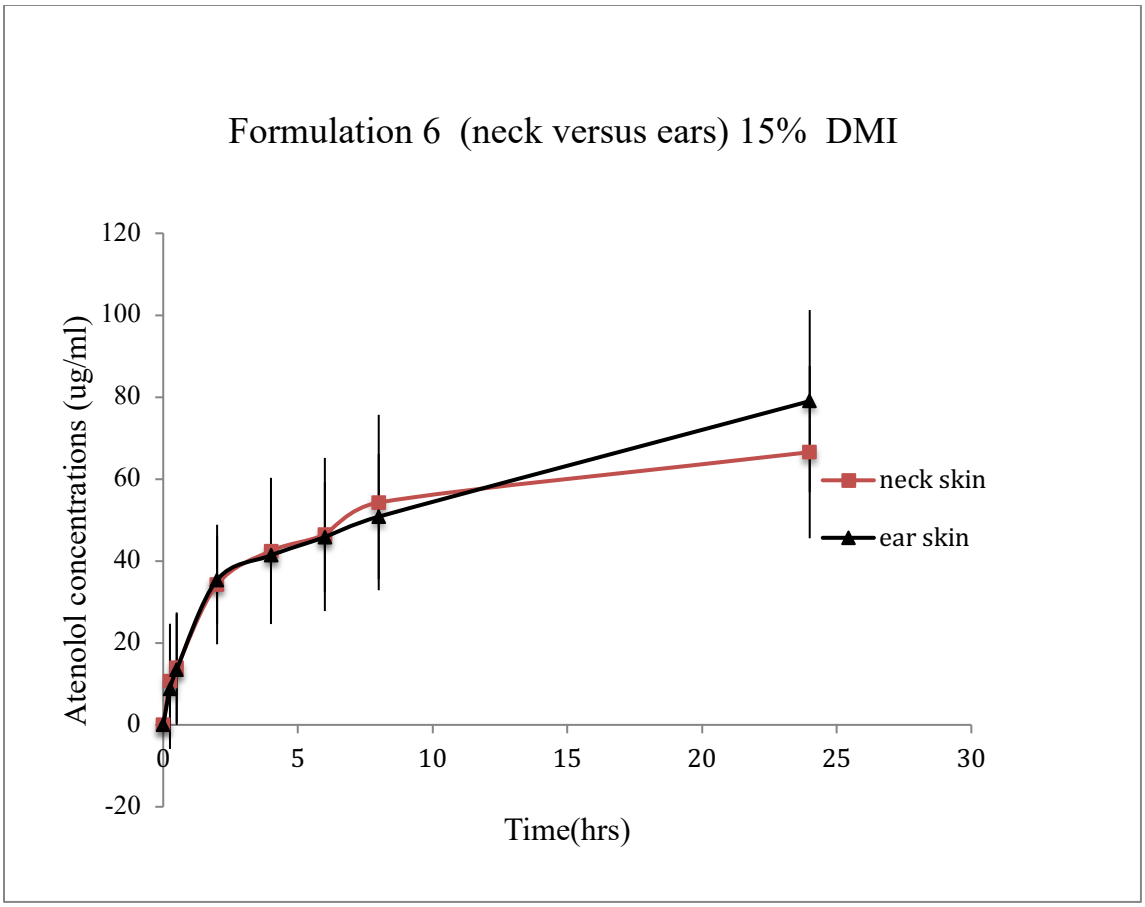


Figure 1.9 Comparison of atenolol concentration time curves for formulation 6 through cat neck skin versus cat ears skin.

DISCUSSION

As illustrated in this chapter, the optimization of an atenolol transdermal formulation was developed by modifications of the formula that was tested in a previous study (Mac Gregor J M et al, 2008). The addition of chemical enhancers which are commercially available (as required by the veterinary medicine legislation) such as surfactants (polysorbate 80), glycerol and propylene glycol also provided enhanced skin permeation of atenolol. Ethanol was added to allow maximum transportation of atenolol through skin. Importantly, dimethyl-isosorbide (DMI) was added at a 15% concentration to further help the transportation of atenolol through cat skin.

The optimized general formula was a 1% atenolol clear, transparent gel vehicle. The gel contained propylene glycol and glycerin as pharmaceutical excipient (15%) and also contained 5 % surfactant (Polysorbate 80) to improve drug permeation through the skin barriers. Ethyl alcohol was added at 10%. The foundation of the gel was 0.75% carbomer that was neutralized with 0.45% triethanolamine producing a pH of less than 7.4

The higher transit of drug through synthetic membrane was due to the larger pore size of the membrane compared to cloned epidermis and cat skin. The pore size of the synthetic membrane (0.45 μm) to the molecular size of atenolol is significantly larger allowing greater amount of atenolol to diffuse through. Cloned human epidermis and cat skin have stronger barrier properties than of that of the synthetic membrane with cloned human skin being the most resistant atenolol diffusion. This may be due to the fact that the epidermis of the cats is a third of the thickness of human epidermis. The atenolol concentrations

transported through the cloned human epidermis were as following: 5.05, 5.878 and 16.26 (ug/ml) for F3, F5 and F6 respectively through 8 hours. Deterioration of the cloned human epidermis after 8 hours allowed large amounts of atenolol to perfuse through the membrane increasing the rate of atenolol flux.

Permeation of atenolol through cat skin over 24 hours was 45.61, 56.1, and 76.56 ug/ml for formulations F3, F5 and F6 respectively. These results are consistent with cat skin being one-third the thickness of human skin. The results showed that Formulation 6 had a greater amount of atenolol that permeated through human epidermis and cat skin with a more consistent drug release.

The aid the percutaneous drug enhancers have on atenolol diffusion through cat skin increased penetration of atenolol through cat skin by 6.8, 7.4, and 8.3 times. The solubility of atenolol is 26.5 mg/ml in water. The solubility of atenolol was determined to be slightly greater than 100 mg/ml that is 4 times greater in solubility in the co-solvent system of Formulation 6 than water. The increase in atenolol solubility in the co-solvent does not fully account for the increase in permeation of atenolol through cats skin revealing that the use of penetration enhancers are needed to produce sufficient atenolol penetration.

The transdermal drug delivery approach has many advantages over conventional oral treatment. These include the avoidance of first pass metabolism; treatment can be quickly started or stopped. The rate of systemic delivery can be more uniformly maintained and sometimes provide a sustained release of the drug and better patient compliance. The transdermal route provides the clinician with a possible extra therapeutic option for patient therapy. Disadvantages include the potential for localized irritation or allergies and difficulties associated with the time necessary for the drug to diffuse through the skin

(Blank et al 1969). Atenolol's chemical mass (226.336 g/mole) and the daily dose for cat, which is around 25 mg/ day appears to make atenolol a good candidate for transdermal delivery. Atenolol is a small molecule but its polarity and solubility is hydrophilic in nature which may be of concern in limiting its capability to cross the epidermis. Our results imply that atenolol can perfuse through cat skin with the aid of the addition of penetration enhancers.

Drug transportation through the different types of barriers varies according to the change in compositions and percentages of the gel ingredients and on the drug molecule type. The addition of commercially available chemical enhancers such as surfactants, glycerol or propylene glycol provided an enhanced skin permeation of atenolol. Additionally, the percentage of ethanol added to this study's test formulation allowed maximum transportation of the drug through the skin by enhancing penetration beyond the reported results in the previous study (Mac Gregor JM et al., 2008). Polysorbate 80 as a non-ionic type of surfactants was chosen. This is the most commonly used surfactant for transdermal delivery. Polysorbate 80 appears to be less irritating and better tolerated by the skin compared to the other surfactants because of its lower critical micellization concentrations produced for emulsification that may work as a transporting aid and provide enhancement for atenolol permeation through cat skin. DMI also provides a synergistic effect with the surfactants to help increase drug transportation across skin allowing an optimal topical formulation to be achieved.

Studies are often performed to predict how a delivery system might work in an ideal situation as well as give some indications of its in vivo performance since drug release dictates the amount of drug available for absorption. All the test formulations exhibited

zero order release on average for atenolol diffusion through the membranes tested. Animal skin has different (generally higher drug permeability) compared to human skin. Fortunately, the cat epidermis is one third the thickness of a human epidermis and has a pH that ranges from 6.5 to 7.5 (Karen L, 2006). This difference may be a key to formulate an effective transdermal gel.

Examining the results; the synthetic membrane diffusion profiles of the applied formulations suggested that the highest penetration of atenolol was obtained with Formulation (3) where the concentration of the penetration enhancers were as follows: 15% for both glycerol and propylene glycol, and 5% Polysorbate 80 and no DMI was added to this formulation. Formulation 6 with 15% DMI was the next best formulation in terms of drug diffusion followed by formulation 5 (10% DMI). The addition of DMI did provide an enhancement to atenolol penetration for Formulations 5 and 6 but did not with Formulation 3. Diffusion of F3 through synthetic membrane is probably related to atenolol solubility only in the base. Emulsification tends to increase solubility of drugs. The results of the experiment with cloned human epidermis gave permeation profiles of the three best formulations F3, F5 and F6. Formulation 6 with 15% DMI produced the highest concentration of the drug penetrating through the cloned human and cat epidermis. In general, the total atenolol concentrations that diffused though cloned epidermis skin was less than that obtained with synthetic membrane. This is expected as the Stratum Corneum of cloned human skin has a complicated structure and possesses a barrier property similar to human skin. Atenolol diffusion profiles of the formulations F3, F5 and F6 through freshly collected cat skin produced the highest observed concentration of atenolol 2 hours after gel application from formulation F6 (35.5 ug/ml) (the First distinct Flux) and after 24

hours allowed 76.56 ug/ml to diffuse through cat skin. Formulation 5 allowed 31.5 ug/ml of the drug through cat skin after 2 hours and 56.1 ug/ml after 24 hours while Formulation 3 (with 0% DMI) atenolol diffusion through cat skin was the lowest after 2 hours at 28.98 ug/ml and 45.6 ug/ml after 24 hours.

An important finding is the optimal concentration of Carbomer 934 is 0.75%. Applying atenolol gel made with 1% carbomer 934 revealed the drug remained inside the gel structure preventing its diffusion through the different membranes as no drug was detected in the receiver compartments for formulations F7, F8 and F9.

The optimized formulation determined by diffusion studies had the following formula and was chosen for the feline in-vivo transdermal atenolol administration study.

5% Tween 80

15% Propylene glycol

15% Glycerol

10% Ethanol

15% DMI

0.75% Carbomer (934).

0.45 Triethanolamine.

The concentration of atenolol in the receiver compartment after 2 hours application to cat skin was 35.5 ug/ml. The Franz cell volume is 14 ml which gives 491.7 ug/ml. The therapeutic concentration of atenolol is 260 ng/ml (Quinone et al., 1996). Atenolol has a volume of distribution of ~1000 ml in cat suggesting that therapeutic concentrations of

atenolol can be attained using the above mentioned formula. Also, upon comparison of the two sites of drug application (neck versus ear) it was found that the ear permits more drug penetration through cat skin.

Statistical analysis using the t-test showed that formulation F3 is significantly different from formulations F5 and F6 ($p<0.05$). The similarity factor f_2 is routinely used to assess if two dissolution curves are the same (Diaz D et al., 2016). As drug diffusion curves are remarkably similar to dissolution curves a similarity factor analysis of the drug diffusion data through the membranes was performed. The similarity factor tests did not indicate any differences between the three best formulations.

CONCLUSION

The ingredients in the optimized formulation produced the enhancement that was desired to obtain a sufficient amount of atenolol being diffused through the cat skin. The enhancers that were used in the optimized formulation were powerful and improved the atenolol permeation more than the increase in atenolol solubility in the formulation. The enhancers used 5% Polysorbate 80 (a strong surfactant), Propylene glycol, Glycerol added at 15 % for each as both enhancers are strong solvent and can provide occlusive effects that helps atenolol penetration by increasing the skin hydration. Ethanol was Also added as a strong solvent enhancer at 10% concentration. Dimethyl-isosorbide (DMI) was added at 15 % concentration which provided more skin mobilization and enable more atenolol penetration. The Formulation was adjusted in terms of its texture by limiting the amount of the Carbomer to (which used as a gel base) to 0.75% so the required amount of softness is obtained. Different atenolol formulations were tested and evaluated to get the most effective formulation. The 6 formulations that were designed for the current study were tested on the different membranes (synthetic, cloned human epidermis and cat skin). Atenolol concentrations obtained in the receiver compartment was assayed and estimating the total amount of the diffused atenolol through cat skin it was found that the concentration of atenolol in the receiver compartment 2 hours application of formulation 6 to cat skin was 35.5 ug/ml. Measuring the volume of the receiver compartment of the Franz cell it was found that the Franz cell volume is 14 ml which gives 497 (ug/ml) penetrating over 2 hours. The therapeutic concentration of atenolol is 260 ng/ml (Quinone et al., 1996) and

by considering atenolol has a volume of distribution of ~1000 ml in cat it can be assessed that therapeutic concentrations of atenolol can be attained using the optimized formula.

The feasibility of producing a topical atenolol gel formulation showed that an atenolol transdermal delivery system can be used in veterinary medicine for treatment of feline heart disease. In vivo studies will be illustrated in the Chapter 4 of this thesis and an assessment of the efficacy of the optimized gel formulation to treat feline heart disease will be explained in that chapter.

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CHAPTER 2

DRUG RELEASE MECHANISM OF ATENOLOL

Sumeia M. Mohamed

ABSTRACT

The lipophilic character of the stratum corneum provides the principal barrier to the entry of drug molecules into the body. The stratum corneum forms the outermost layer of the skin and essentially consisted of multilayers of corneocytes that form the skin integrity. Drugs can be administered either as suspensions or as solutions and the formulation can range in complexity from a gel or an ointment to a multilayer transdermal patch. Consequently, drug release can follow different mechanisms and different drug release models are used to explain the processes of drug release. This chapter describes the theoretical principles used to describe transdermal release and membrane transport models based on the appropriate application of Fick's second law of diffusion that can be used to explain drug release kinetics through the skin's complex biological membrane. Transdermal drug delivery systems are designed to control drug release through the skin to achieve systemic effect, maintaining consistent efficacy and reducing dose of the drug and probably its side effects. This study was conducted to prepare transdermal gel of atenolol with a mixture of strong permeation enhancers to improve skin penetration. The formulations presented in the previous chapter were examined by various kinetic models such as Zero-order, First-order, Higuchi equation, Korsmeyer–Pappas equation and Hixson–Crowell equation. The results of the percent cumulative drug release was examined in accordance to the kinetic models of Zero-order, First-order, Higuchi equation, Korsemeyer–Pappas equation and Hixson–Crowell equation. In Zero order, r^2 was calculated for each formulation according to the different kinetic models the values of regression coefficient was r^2= 0.9012 for formulation 3, Formulation 5 r^2= 0.9137 and

Formulation 6 r^2= 0.92. In First order, r^2= 0.855 for Formulation 3 and r^2= 0.791 for formulation 5 and r^2= 0.816 for formulation 6. In Higuchi model graph was plotted between cumulative percentage drug released versus square root of time for each formulation through cat skin and it was not linear and the value of regression coefficient was r^2= 0.607 for formulation 3, and r^2= 0.739 for formulation 5 and formulation 6 value of regression coefficient was r^2= 0.701. In Hixon-Crowell model, the value of regression coefficient was r^2= 0.541 for formulation 3 and r^2= 0.593 and r^2= 0.653 for formulation 6. The study showed synergistic effects of the different enhancers on drug release which was explained best with a zero order model for drug release. The Korsmeyer-Peppas n value for the first phase was 0.8571 for the initial burst flux indicating a fickian diffusion process of a drug solution. The n value for the second flux phase was 0.2616. This indicates that dissolution of the drug from the drug particle occurs before diffusion through the cat skin. Erosion of the drug particle over may also be involved in this process.

INTRODUCTION

Drug release mechanisms have been an extensively researched topic in the field of drug delivery for several decades. Creative formulations with various complexity due to advancement in designs and material production have been introduced into the drug delivery development systems, resulting in improved clinical applicability and quality of life.

The term Drug Release is used to describe the process of drug solutes migration from the initial position in the drug formulation to the formulation's outer surface and then to the release medium (Langer R, 1990).

Factors that affect drug release are complex such as structural characteristics of the material system, the physicochemical properties of the solute molecules and the release environment affect drug release mechanisms. The interactions between these different factors also can affect the drug release pattern. The previously mentioned driving factors can be classified as following: 1. Release medium: pH, ionic strength and temperature, 2. Composition of the material matrix; its structure and degradation ability, 3. Drug molecules physical properties: e.g. Stability, solubility, charges and the solute interaction with matrix (Yao F and Weiyuan K, 2010).

This chapter presents various models used in illustrating the mechanisms of drug release from different matrices. The goal of this chapter is to identify the atenolol release pattern from the newly formulated gel.

MATERIALS AND METHODS

Kinetic models

There are a number of drug release kinetic models that have been introduced to understand the drug release mechanism and help scientists to predict the *in-vivo* drug profile data. Drug dissolution from solid dosage forms has been described by some kinetic models. The models have also been applied to semisolid and gel formulations. These models are:

1- zero-order kinetics,

2- first order kinetics,

3- Higuchi model

4- Hixson-Crowell (Kalam MA et al., 1997).

5- Korsmeyer- Peppas Model.

Process of drug release:

Drug release processes that control drug molecule release from a formulation are diffusion, erosion, and dissolution. Mixed mechanisms sometimes control the drug release. Drug release may involve both diffusion and dissolution processes (Ofoefule SI and Chukwu A, 2002).

Parameters that affect drug permeability include:

1. The permeability of the drug vehicle to dissolution medium.
2. The solubility of the drug in the dissolution medium.
3. The molecular size of the drug may affect drug release processes. Insoluble drug vehicle can form an impermeable surface that retards drug release.

Leaching in the dissolution medium

Drug release from matrices may involve processes of diffusion, erosion, dissolution and leaching with subsequent dissolution (Kalam MA et al., 1997).

Some drugs leach into dissolution medium. Presence of pores, cracks and inter granular spaces can enable the dissolution medium to enter the drug matrix system. The changes in the interspaces of the matrix control the infiltration rate of the dissolution fluid into the drug matrix (Tahara K et al., 1225). Drug release also may follow mixed mechanism of release. Sometimes it may involve both diffusion and dissolution controlled processes (Shah SU et al., 2011).

Drug release modelling

Drug dissolution from solid dosage forms has been described by some kinetic models which include zero-order kinetics, first order kinetics, Higuchi model and Hixson-Crowell. The mechanisms of drug release from a matrix can be interpreted using these models: Weibull model, Baker-Lonsdale model, Korsmeyer-Peppas and Ritger-Peppas model and Hopfenberg model (Kalam MA et al., 1997, Shah SU et al., 2011).

Zero order mechanism

The zero order rate: The drug release rate is independent of its concentration (Kalam MA et al., 1997).

$C=K_o t$

Where, K_o is zero-order rate constant (units of concentration/time).

t is the time.

A drug release system that follows this model produces a linear relation when plot the amount of drug released versus time will produce a linear relationship.

Zero order mechanism means that the system releases the same amount of drug per unit time in a continuous fashion for the major portion of time of the drug release. Zero order mechanism is the method of drug release that is intended to produce a sustained action. Matrix tablets with low soluble drugs exhibits a zero order release (Varles CG et al., 1995).

First order mechanism

Systems with water soluble drugs usually follow this model (Mulye NV et al., 1995).

The first order kinetics was first applied for drug dissolution studies by Gibaldi and Feldman in 1967. Wagner also applied this mechanism in 1967 (Gibaldi M and Feldman S, 1967) (Wagner JG, 1967).

Unlike the zero order model, in the First order model, the rate of drug release is dependent on the drug concentration. (Kalam MA et al., 1997). Two formulas of the first order equation are

$\text{Log } C_t = \text{Log } C_o - K_1 t / 2.303$

$\text{Log} C_o - \text{Log } C_t = Kt / 2.303$

Where, C_t is the amount of drug released in time t,

C_o is the initial concentration of drug.

K_1 is first order constant.

Higuchi models

This model is designed to study the release of water soluble and low soluble drugs incorporated in semisolid and solid matrices. Higuchi in 1961 and in 1963 developed these models (Higuchi, 1961, Higuchi, 1963).

The relation is shown below as

$Q = [D (2C-C_s) C_s t]^{1/2}$

Where Q is the amount of drug released in time t per unit area.

C is the initial drug concentration.

Cs is the drug solubility in the matrix media

As C >> Cs

D is the diffusivity of drug molecules in the matrix substance.

Simplifying the equation becomes,

$Q = K_H t^{1/2}$

Where K_H is the Higuchi dissolution constant.

Higuchi describes drug release as a diffusion process based in the Fick's law. In this model the rate of release is proportional to the square root time. Therefore, for diffusion controlled process a plot of Q versus square root of time gives a linear relation. To establish weather mixed kinetics of drug release exists, an integral form of Higuchi equation is stated as

$LogQ=logK_H+1/2logt$

when the logarithm plot approaches 0.5, the diffusion process becomes the main process of drug release (Kalam M A et al, 2007) (Ofoefule SI and Chukwu, 2002).

Hixson-Crowell cube root law: The Hixson-Crowell cube root law is presented as

$Q_0^{1/3} - Q_t^{1/3} = K_{HC}t$.

The model describes the release from systems where a change in surface area and diameter occurs to particles or tablets (Hixon AW and Crowell, 1930) (Shoaib HM et al., 2006).

Where, Q_t is the remaining amount of drug in the dosage form at time t.

Q_o is the initial amount of the drug in tablet.

K_{HC} is the rate constant for Hixson-Crowell rate equation.

A graphical representation of the cube root of the amount remaining versus time will be linear if the equilibrium conditions are not reached and if the geometrical shape of the dosage form diminishes proportionally overtime (Cube root of initial drug load minus cube root of percent drug remaining) are plotted against time (hour) to demonstrate the Hixson Crowell plot (Rahman M et al., 2011).

This model is used by assuming that release rate is limited by the drug particles dissolution rate and not by diffusion (Kalam M A et al., 2007).

Korsmeyer-Peppas models and Ritger-Peppas

Ritger and Peppas (1987) and Korsmeyer and Peppas (1984) developed an empirical equation to analyze both Fickian and non-Fickian release of drug from swelling as well as

non-swelling polymeric delivery systems (Korsmeyer RW and Peppas NA, 1983) (Ritger PL and Peppas NA, 1987b).

The equation is

$Mt/M\infty = Kt^n$ The logarithm form of equation could be written as:

Log (Mt /M∝) = Log k + n Log t where Mt/M∝ is fraction of drug released at time t,

n is diffusion exponent indicative of the mechanism of transport of drug through the polymer, K is kinetic constant (having units of t^{-n}) incorporating structural and geometric characteristics of the delivery system.

For Ritger-Peppas models, the release exponent $n \leq 0.5$ for Fickian diffusion release from matrix (non swellable matrix), $0.5 < n < 1.0$ for non-Fickian release (anomalous), This means that drug release followed both diffusion and erosion controlled mechanisms and $n = 1$ for zero order release, (Korsmeyer RW and Peppas NA, 1983) (Singh J et al., 2011). Also, $0.45 < n < 1.0$ for non-Fickian release (anomalous) from cylinders (non swellable matrix) and $0.43 < n < 1.0$ for non-Fickian release (anomalous) from non swellable spherical samples.

For Korsmeyer-Peppas models, the release exponent $n \leq 0.45$ for Fickian diffusion release and $0.45 < n < 0.89$ for non-Fickian release (anomalous).

In diffusion processes a cross membranes $n > 0.45$ is a dissolusion/ erosion release of drug for diffusion where $n < 0.45$ is for diffusion of drug in a solution through a membrane.

Weibull model

The Weibull equation expresses the accumulated fraction of drug ‘m’ in solution at time t as:

$M = M_o [1- \exp [-(t-Ti/a)^b]$

Where M is the accumulated fraction of drug in solution at time t. 'a' is the scale parameter which defines the time scale of the process. Ti is the location parameter, represents the lag time before the onset of the dissolution or release process and in most of the cases will be zero. The shape parameter, b, characterizes the curves as either exponential (b=1), S-shaped (b>1) or parabolic (b<1) [19]. The previous equation can be rearranged as:

Log [ln - (1-m)] = b Log (t-Ti)-log a

Graphical representation of log [-ln (1-m)] versus time t gives a linear relation.

Shape parameter (b) is obtained from the shape of the line and the scale parameter (a) can be estimated from the ordinate value (1/a) at time t =1 (Kalam M A et al., 2007).

Baker-Lonsdale model

Baker-Lonsdale in 1974 developed the model from the Higuchi model and describes the controlled release of drug from a spherical matrix that can be represented as:

$3/2\ [1-(1-Mt/M\infty)^{2/3}]-Mt/M\infty = (3DmCms) / (r_o^2\ C_o)\ Xt = Xt$. Where Mt is the amount of drug released at time t. M∞ is the amount of drug released at an infinite time.

Dm is the diffusion coefficient, Cms is the drug solubility in the matrix. r_o is the radius of the spherical matrix.

C_o is the initial concentration of the drug in the matrix (Baker RW and Lonsdale HS, 1974).

Hopfenberg Model

(Hopfenberg,1976) and (Katzhendler et al., 1997) developed a general mathematical equation describing drug release from slabs, spheres and infinite cylinders displaying heterogeneous erosions as:

$Mt/M\infty = 1 - [1-K_o t/C_o a_o]^n$ Where Mt is the amount of drug dissolved in time t.

$M\infty$ is the total amount of drug dissolved when the dosage form is exhausted.

$Mt/M\infty$ is the fraction of drug dissolved. K_o is the erosion rate constant.

C_o is the initial concentration of drug in the matrix and a_0 is the initial radius for sphere or cylinder or the half-thickness for a slab. The value of n is 1, 2, and 3 for a slab, cylinder and sphere respectively (Kalam M A et al., 2007) (Costa P and Sousa Lobo JM, 2003) (Katzhendler I et al., 1997).

Power law

Power law is a semi-empirical equation that describes drug release from polymeric system as shown below

$At/A\infty = kt^n$

Where At and $A\infty$ are the absolute cumulative amount of drug released at time 't' and at time infinity respectively, 'k' is a constant incorporating structural and geometric characteristics of the device and n is the release exponent, indicative of the drug release mechanism (Kalam M A et al 2007).

The exponent n between 0.5 and 1.0 for anomalous release mechanism, while n = 1.0 for zero-order kinetics and n = 0.5 for diffusion controlled drug release.

Drug release kinetics can be determined using zero-order, first order, Higuchi and Hixson-Crowell models, while the mechanisms of drug release can be determined using Weibull model, Baker-Lonsdale model, Korsmeyer-Peppas or Ritger-Peppas model. For each model the slope (n), regression coefficient (r^2) and rate constant (k) are graphically

determined and are used to predict the kinetics and mechanisms of drug release from matrices.

Fick's law

Fick's law of diffusion describes how particles under random thermal motion tend to move from a region of higher concentration to a region of lower concentration. Mathematically the diffusion is characterized by Fick's law which states that the diffusion flux is proportional to the concentration gradient:

$F = - D \Delta C$

where C is the concentration of the diffusing particles.

F is the diffusion flux (particles per square meter per second).

D is the diffusion constant, which has units of cm^2 per second.

Fick's Law of diffusion describes the time course of the transfer of the solute molecules between two compartments that are separated by a thin membrane, given by

$F = - D \, dc/dx$. Where c = concentration, D = diffusion coefficient, dx = membrane thickness

Fick's First Law, for the flux due to diffusion across a plane in one dimension, is

Net flux per unit area.

RESULTS AND DISCUSSION

Release Mechanism of Optimized Formulation

The determination of the drug release mechanism from the prepared transdermal formulations including the optimized formulation (F6), the diffusion results of the percent drug release were examined in accordance to the different kinetic models such as Zero-order, First-order, Higuchi equation, Korsmeyer–Pappas equation and Hixson–Crowell equation. The regression coefficient r^2 value nearer to 1 indicated the model fitting the release mechanism. Other formulations were examined but only the data of formulations 3-6 is presented as the results were similar to formulation 6 results.

Formulation code	Hixson Crowell Slope	Hixson Crowell r^2	Zero order Slope	Zero order r^2	First order slope	First order r^2
Formulation 3	0.0261	0.541	6.49	0.9012	0.01703	0.8556
Formulation 5	0.0573	0.593	5.82	0.9173	0.0156	0.79
Formulation 6	0.052	0.653	8.353	0.920	0.0163	0.816

Table 2.1: The slope of the drug diffusion versus time profile along with the r^2 values of various formulations obtained after fitting the drug release data to various release kinetic models; shows zero order model best fitted the data.

Formulation no	Zero order r^2	first order r^2	r^2(Higuchi)
F3	0.9012	0.855	0.607
F5	0.9173	0.791	0.739
F6	0.920	0.816	0.701

Table 2.2: Kinetic parameters of the release curve showing best fit with higher correlation with the zero order equation for almost all the formulations (3, 5 and 6).

As shown in table 2.1 the zero order R^2 calculated for each formulation according to the different kinetic models were quite similar. The values of regression coefficient r^2 was = 0.9012 for Formulation 3, Formulation 5; the r^2= 0.9137 and Formulation 6 r^2= 0.92. In first order model the regression coefficient r^2= 0.855 for Formulation 3 and r^2= 0.791 for formulation 5 and r^2= 0.816 for formulation 6. In the Higuchi model the cumulative percentage of drug released versus square root of time for each formulation through cat skin was plotted but was not linear and the value of regression coefficient was r^2= 0.607 for formulation 3, and r^2= 0.739 for formulation 5 and formulation 6 value of regression coefficient was r^2= 0.701. In Hixon-Crowell model, the value of regression coefficient was r^2= 0.541 for formulation 3 and r^2= 0.593 and r^2= 0.653 for formulation 6.

Higuchi model evaluation

Since atenolol is water soluble, further testing with Higuchi model was performed. Figures 2.1 to 2.3 show Higuchi plots of atenolol diffusion through cat skin. These graphs show that atenolol release pattern from the gel does not obey this model. Attempts were made to determine if other mechanisms for drug release based on the other models could be applied to drug release from topical atenolol gels. The results were less than adequate at explaining drug release patterns from the gel. Figure 2.1 presents the Higuchi plot for Formulation 6. The plot doesn't show the linearity that is required to explain the drug release pattern although it is the straightest plot compared to the Figure 2.2 for Formulation 3 and Figure 2.3 for Formulation 5 for the Higuchi model.

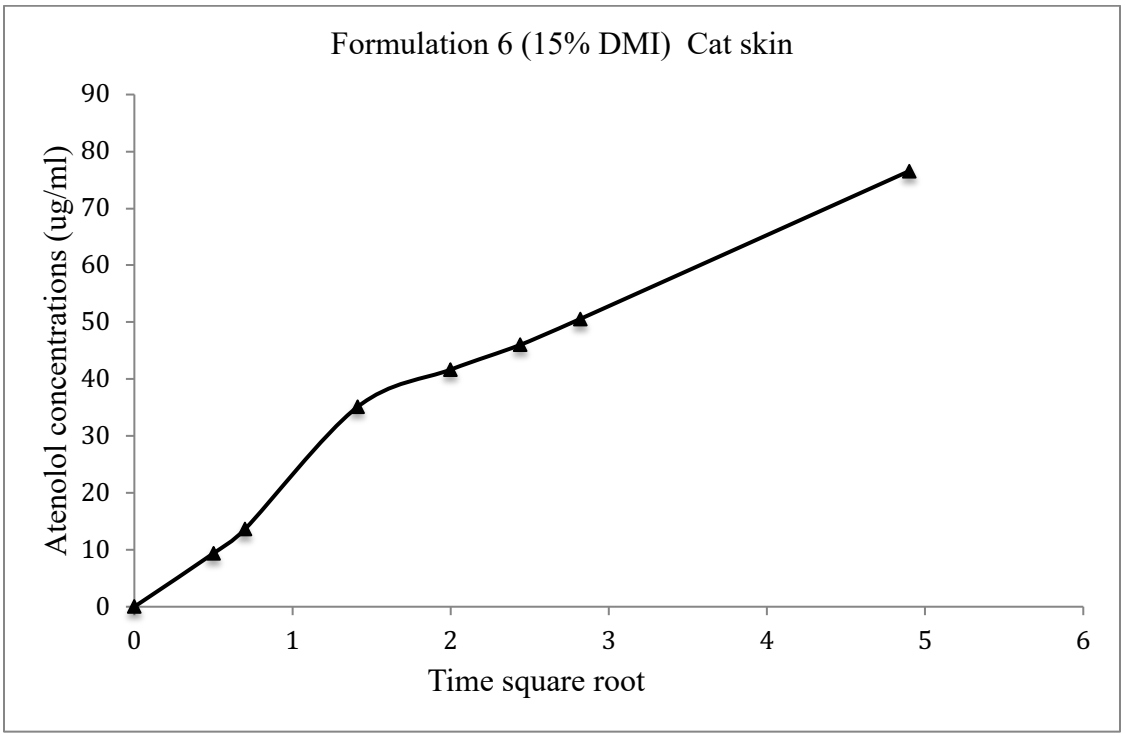

Figure 2.1 The atenolol average concentration versus the square root of time (formulation 6) (Higuchi plot**).**

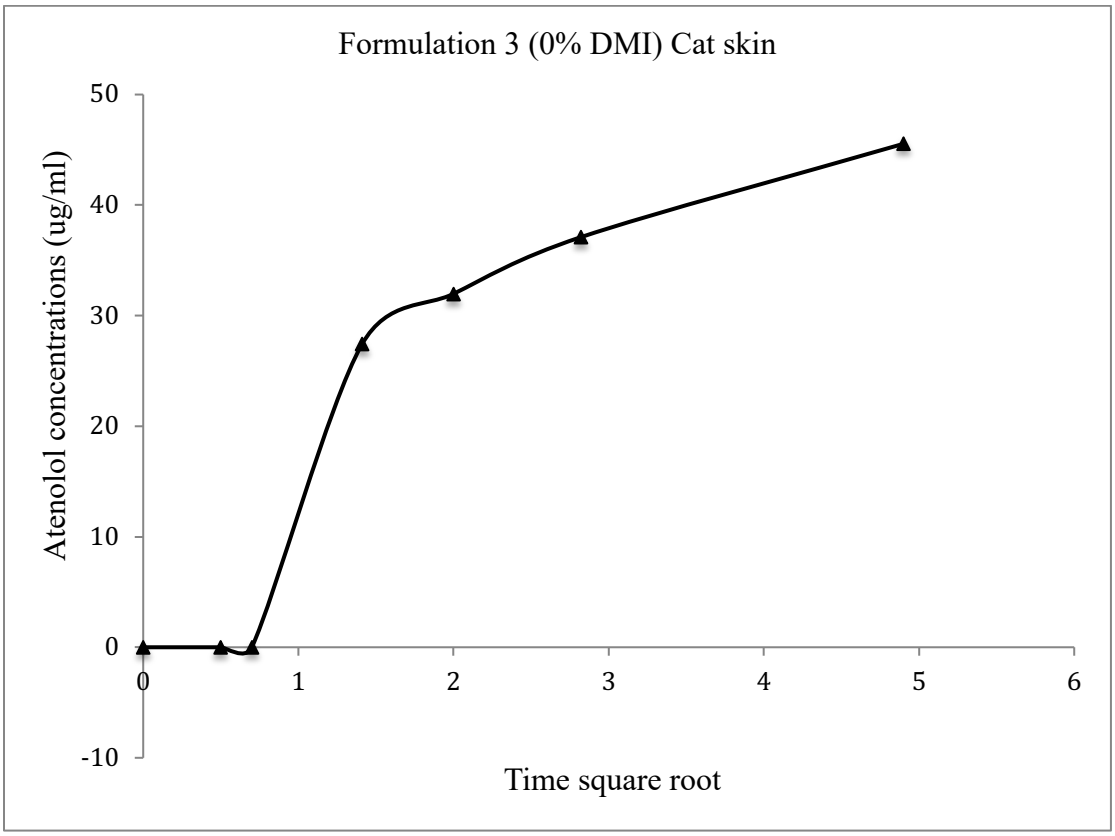

Figure 2.2 The atenolol average concentration versus the square root of time (formulation 3) (Higuchi plot**).**

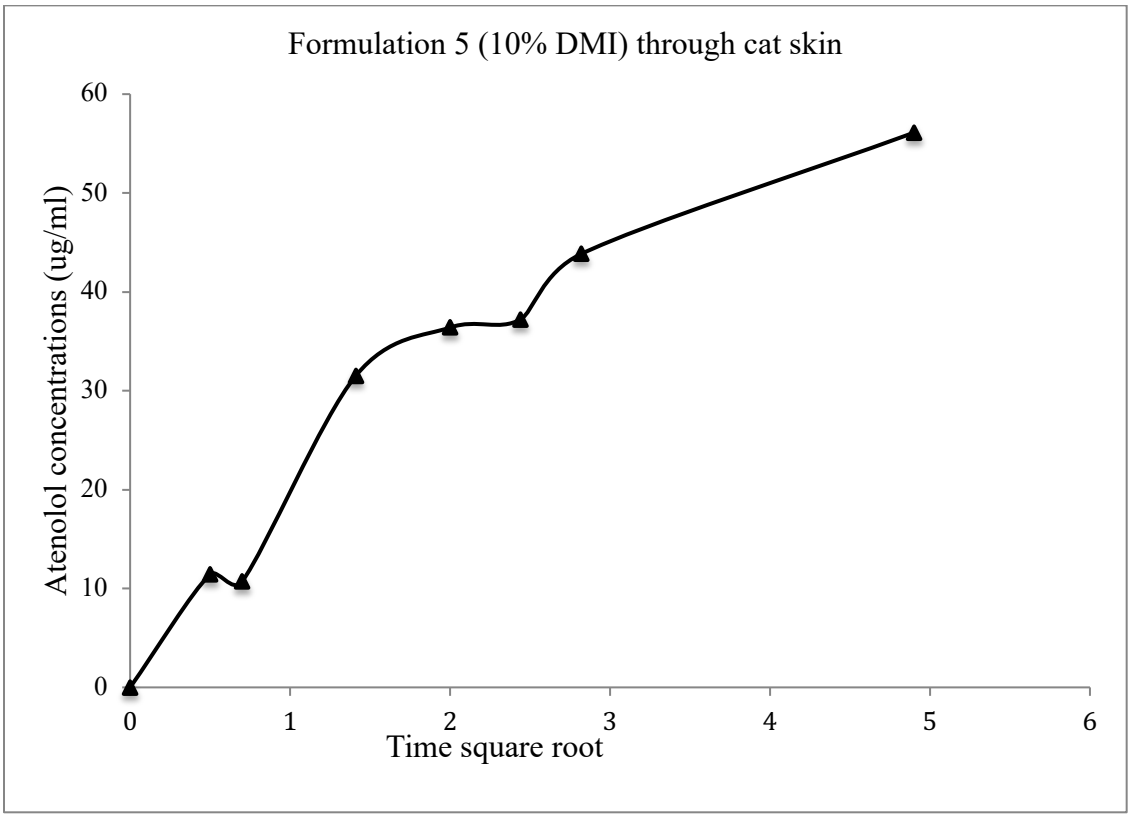

Figure 2.3 The atenolol average concentration versus the square root of time (formulation 5) (Higuchi plot).

Atenolol release pattern (Fick's law with double phases)

Figure 2.4 demonstrates two phases for the drug release. The initial diffusion of atenolol through cat skin is similar to a Burst Flux followed by a Sustained Flux for the rest of the drug diffusion time profile. All atenolol gel formulations produced an initial rapid burst of diffusion through the test membranes followed by a significantly slower flux of atenolol from test formulations (Tables 2.3 and 2.4).

Formulation 6 gave the highest burst of atenolol diffusion through cat skin for the initial two hours (35.5 ug/ml) followed by a zero-order sustained diffusion of atenolol that lasted up to twenty-fours after atenolol application (24 hours atenolol concentration 76.56 ug/ml). This biphasic diffusion profile appeared to give the most promise for topical atenolol treatment for cats with hypertrophic obstructive cardiomyopathy. The initial flux of atenolol across cat skin gave the highest predicted atenolol concentrations reaching a value near 340 ng/ml that would well above the reported therapeutic concentration of 260 ng/ml.

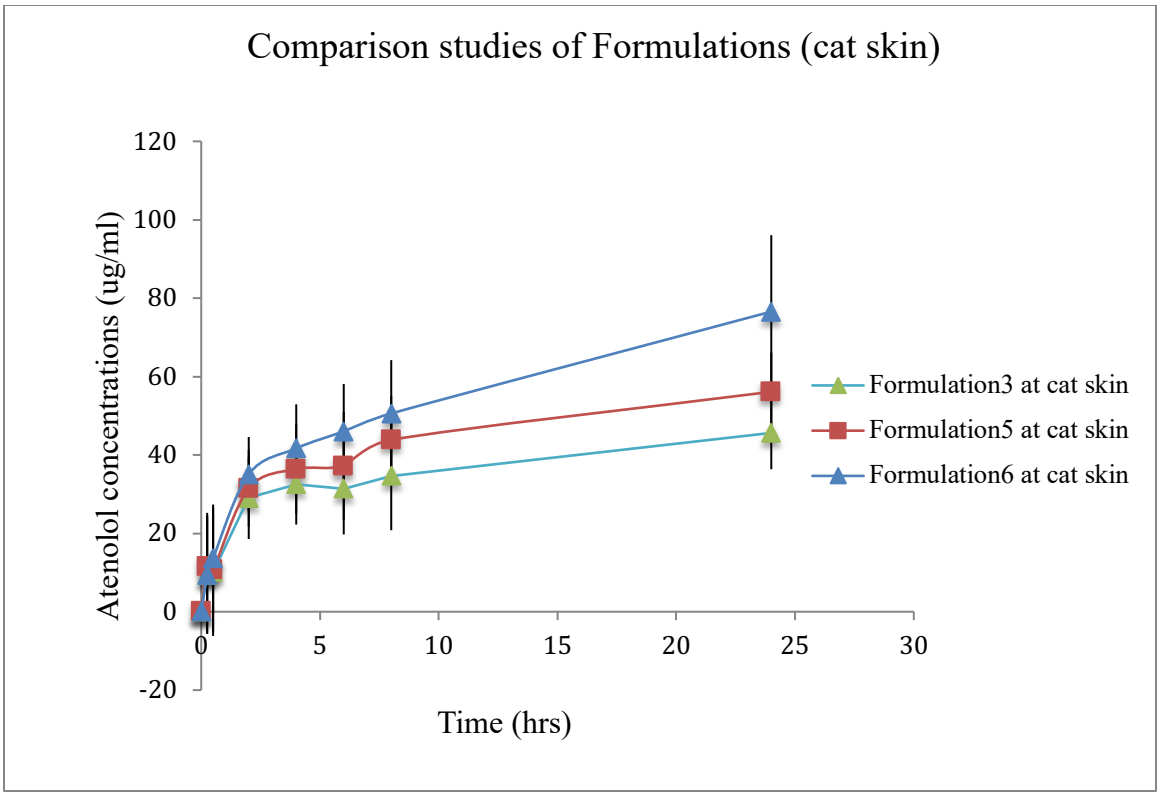

Figure 2.4 Permeation of atenolol from formulations 3, 5, and 6 through excised cat skin.

Franz cell no	Formulation 3 First Flux (J_1) (ug/cm²/hr)	Formulation 5 First Flux (J_1) (ug/cm²/hr)	Formulation6 First Flux (J_1) (ug/cm²/hr)
FRANZ1	27.22	62.248	66.06
FRANZ2	56.73	77	71.74
FRANZ3	7.67	58.22	73.98
FRANZ4	13.245	74.93	75.07
FRANZ5	13.326	62.31	73.659
FRANZ6	15.597	22.195	18.805
FRANZ7	14.47	15.361	16.516
FRANZ8	16.025	15.52	18.084
FRANZ9	14.838	16.57	18.26
FRANZ10	13.857	18.18	17.568
FRANZ11	12.53	13.24	16.93

Table 2.3 The initial flux (J_1) for each formulation in cat skin.

FRANZ NO	FORMULATION 3 FLUX (J_2)	FORMULATION 5 FLUX(J_2)	FORMULATION 6 FLUX (J_2)
FRANZ1	1.07	1.362	1.64
FRANZ2	1.08	1.19	1.43
FRANZ3	0.97	1.396	1.07
FRANZ4	1.369	1.224	3.109
FRANZ5	1.515	1.5	3.78
FRANZ6	1.7	1.634	3.5012
FRANZ7	1.909	2.48	2.66
FRANZ8	1.94	2.12	2.9
FRANZ9	1.98	2.27	3.211
FRANZ10	1.89	2.31	3.405
FRANZ11	1.06	1.98	3.7

Table 2.4 The second Flux (J_2) values of the selected formulations through cat skin

Korsmeyer-Peppas Model Evaluation

Upon testing the atenolol data using the Korsmeyer-Peppas model the following charts were produced. Figure 2.5 demonstrates the Korsmeyer-Peppas model on the both phases of atenolol diffusion. Figure 2.6 and 2.7 shows the Korsmeyer-Peppas model on burst and sustained phases of atenolol diffusion respectively.

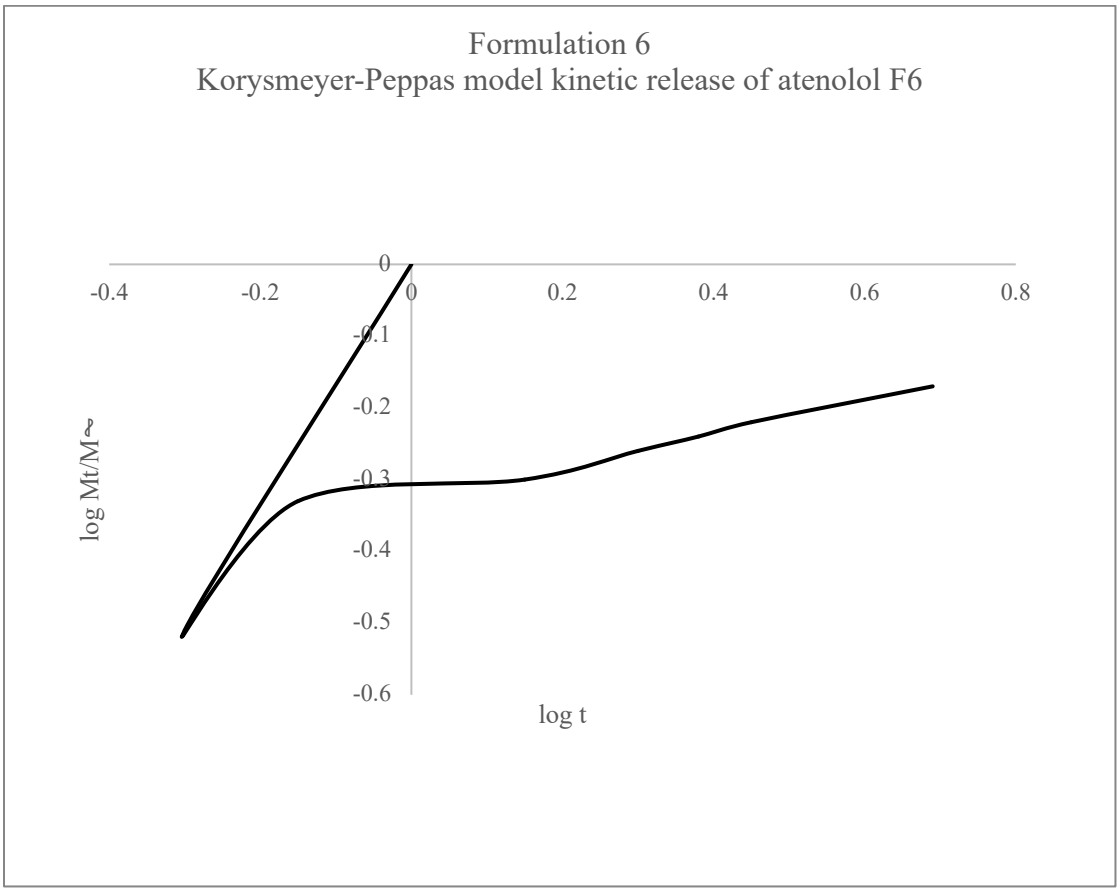

Figure 2.5 The Korysmeyer-Peppas model kinetic release of atenolol from formulation 6.

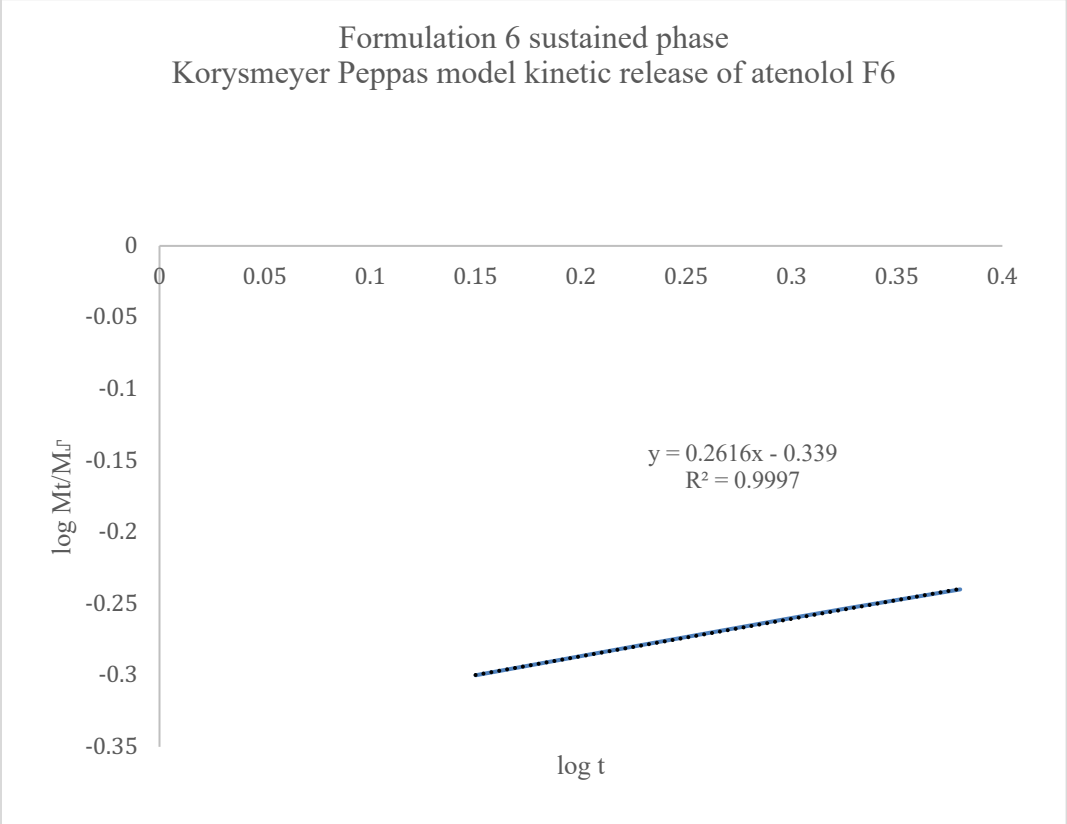

Figure 2.6. The (Sustained phase) Korysmeyer-Peppas model kinetic release of atenolol from formulation 6.

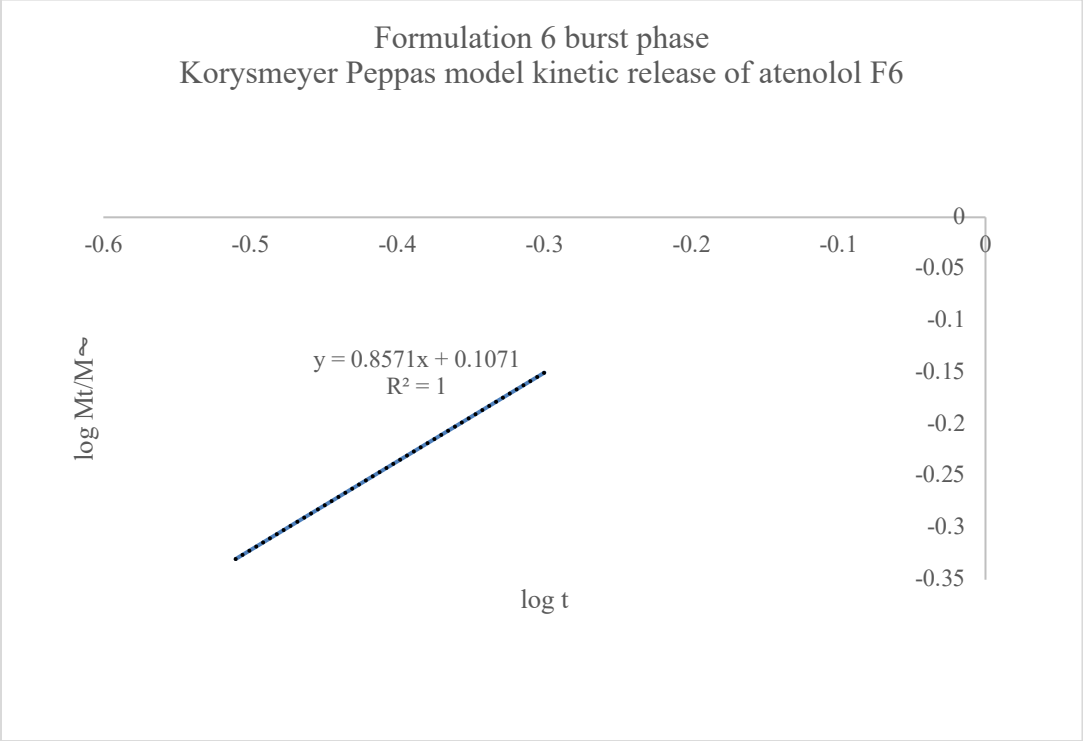

Figure 2.7 The (Burst Phase) Korysmeyer Peppas model kinetic release of atenolol from formulation 6.

Several models were tested to determine the mechanism of drug diffusion through cat and the various membranes figures 2.5, 2.6 and 2.7. A zero order Fick's law of diffusion gives the best model to explain atenolol diffusion through the membranes tested. The model was especially capable in deciphering the type of diffusion seen through cat skin. Permeation of atenolol through cat skin followed a biphasic diffusion process. The initial diffusion was a burst of atenolol penetration through the membrane followed by a slower rate of drug permeation. Both phases of drug penetration followed a zero-order kinetic release pattern. The Korsmeyer-Peppas n value for the first phase was 0.8571 for the initial burst flux indicating a fickian diffusion process of a drug solution. The n value for the second flux phase was 0.2616. This indicates that dissolution of the drug from the drug particle occurs before diffusion through the cat skin. Erosion of the drug particle over may also be involved in this process.

CONCLUSION

The results of the cumulative percent drug release were examined in accordance to the kinetic models such as Zero-order, First-order, Higuchi equation, Korsmeyer–Pappas equation and Hixson–Crowell equation. In Zero order, R^2 was calculated for each formulation according to the different kinetic models the values of regression coefficient was R^2= 0.9012 for formulation 3, Formulation 5 R^2= 0.9137 and Formulation 6 R^2= 0.92. In First order, R^2= 0.855 for Formulation 3 and R^2= 0.791 for formulation 5 and R^2= 0.816 for formulation 6. The graph of the data using Higuchi model was plotted. The cumulative percentage drug released versus square root of time for each formulation through cat skin was not linear and the value of regression coefficients of R^2= 0.607 for formulation 3, and R^2= 0.739 for formulation 5 and formulation 6 value of regression coefficient was R^2= 0.701 were considerably less than the R^2 values for the zero-order model. In Hixon-Crowell model, the value of regression coefficient was R^2= 0.541 for formulation 3 and R^2= 0.593 and R^2= 0.653 for formulation 6. The Korsmeyer-Peppas model was especially capable in deciphering the type of diffusion seen through cat skin. Permeation of atenolol through cat skin followed a biphasic diffusion process. The initial diffusion was a burst of atenolol penetration through the membrane followed by a slower rate of drug permeation. Both phases of drug penetration followed a zero-order kinetic release pattern. The Korsmeyer-Peppas n value for the first phase was 0.8571 for the initial burst flux indicating a fickian diffusion process of a drug solution. The n value for the second flux phase was 0.2616. This indicates that dissolution of the drug from the drug particle occurs before diffusion through the cat skin. Erosion of the drug particle over may also be involved in this process.

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CHAPTER 3

COMPARATIVE STUDIES OF ATENOLOL *IN-VITRO* DIFFUSION PROFILES

Sumeia M. Mohamed.

ABSTRACT

Topical administration of atenolol provides an effective means of providing systemic treatment. The aim of this thesis was the development of an effective topical formulation of atenolol that was able to promote drug delivery through skin and provide therapeutic atenolol concentrations. Three selected formulations have been investigated as drug carriers: formulation 3 with no DMI (Dimethyl isosorbide), Formulation 5 with 10% DMI and Formulation 6 with 15% DMI. All the formulations contained polysorbate 80 5%, ethanol 10%, propylene glycol 15% and Glycerol 15%. The effect of the formulation composition on atenolol transdermal penetration has been evaluated for each formulation. The objective of this chapter was the evaluation and characterization of the selected formulations by similarity factor f_2, difference or dissimilarity factor f_2, partial AUC and MDT (Mean Diffusion Time). The formulations were previously subjected to *in vitro* permeation studies through synthetic membrane, cloned human epidermis and freshly collected cat skin. All the formulations markedly ($p < 0.001$) improved the drug amount penetrating through the skin with respect to an aqueous solution. A 6.8 to 9.0 folds increase in atenolol diffusion through cat skin was observed for Formulation 3, formulation 5 and formulation 6. Formulation 6 produced the highest increase in drug penetration through cat skin, probably due to the presence in the vehicle composition of 15% DMI acting as a drug carrier. Formulation 6 with 15% DMI can be considered the most promising for topical atenolol administration to give a systemic therapeutic level of atenolol in sick cats as its values were slightly better.

INTRODUCTION

The food and drug administration (FDA) has included the difference factor f_1, and the similarity factor (f_2) in various guidance documents and can be used as criteria for comparison of drug dissolution profiles. These factors are easy to compute. In addition, they provide a single number to compare two companies' dissolution profile data. These factors are the most widely used for comparison of dissolution profiles recommended by FDA (Thomas H et al., 1998).

The similarity factor (f_2)

(f_2) is a logarithmic transformation of the sum-squared error of differences between the (test) and reference products (R) through all time intervals. Similarity factor (f_2) represents closeness of two given formulations. According to US FDA similarity factor value in the range of 50-100 is acceptable from the equation for the calculation of the similarity factor (Yuksel N et al., 2000). The FDA suggests that for comparative formulations of two dissolution profiles are declared similar if f_2 is between 50 to 100 as determined using the similarity factor formula.

$$f_2 = 50\log\left\{\left(1+\frac{1}{n}\sum\left(R_t - T_t\right)^2\right)^{-0.5} x100\right\}$$

where,

n = number of dissolution sample times.

Rt and Tt = individual or mean percent of drug dissolved at each time point t, for the reference and test product dissolution profiles, respectively (Yuksel N et al., 2000).

Difference Factor 1 (f_1)

Difference factor 1 (f_1) is able to measure the percent of error between two curves. Mathematically it can be calculated by using the following equation

$$f_1 = \left\{ \left[\sum_{t=1}^{n} |R_t - T_t| \right] \Big/ \left[\sum_{t=1}^{n} R_t \right] \right\} \times 100$$

n = number of dissolution sample times.

Rt and Tt = individual or mean percent of drug dissolved at each time point t, for the reference and test product dissolution profiles, respectively.

This factor is used to compare a test and a reference product in terms of percent drug dissolved per unit time. When tests and reference profiles are identical then f_1 value becomes zero. Generally, f_1 range of 0-10 indicates similarity between the two data profiles (Hussain L et al., 2013).

The similarity factor f_2 reaches a 100 when two comparative groups of reference and test are identical and approaches 0 as the dissimilarity increases. The main advantage of f_2 equation is that it is easy to compute and provides a single number to describe the comparison of dissolution profile data. (Costa P and Lobo J, 2001).

Partial AUC (pAUC)

The partial AUC has been used as an alternative measure to the full AUC. When using the partial AUC it considers only the regions of the space where data have been located (generated) (Walter D, 2005).

pAUC metrics provide additional controls over the time course of the PK profile. pAUC provides an indirect method to quantify cumulative amount of mass that is transported through the membrane.

Mean diffusion times (MDT) of atenolol through the membranes were performed to differentiate between atenolol diffusion profiles (Podczeck F, 1993).

MDT = AUMC/AUC

MDT is a method to determine the rate of drug diffusion through the membrane.

All of the above mentioned comparison tests to compare between different atenolol formulations that have been designed and tested in chapter 1 of this thesis were used to determine which formulation produced superior results.

RESULTS

Formulations F3, F5 and F6 were analyzed for comparison where the atenolol concentrations 2 hours after application were considered and compared with to an application of a 1% aqueous solution of atenolol. All the formulations showed improved permeation of atenolol through cat skin ($p < 0.01$) indicating a significant difference over administration of a control 1 % solution of atenolol (Figure 3.1). Atenolol solution applied to ear skin had very low permeation through cat ear skin (4.24 μg/ml). Formulations 3, 5 and 6 increased atenolol by 6.8, 7.4 and 8.3 times greater than the control atenolol solution.

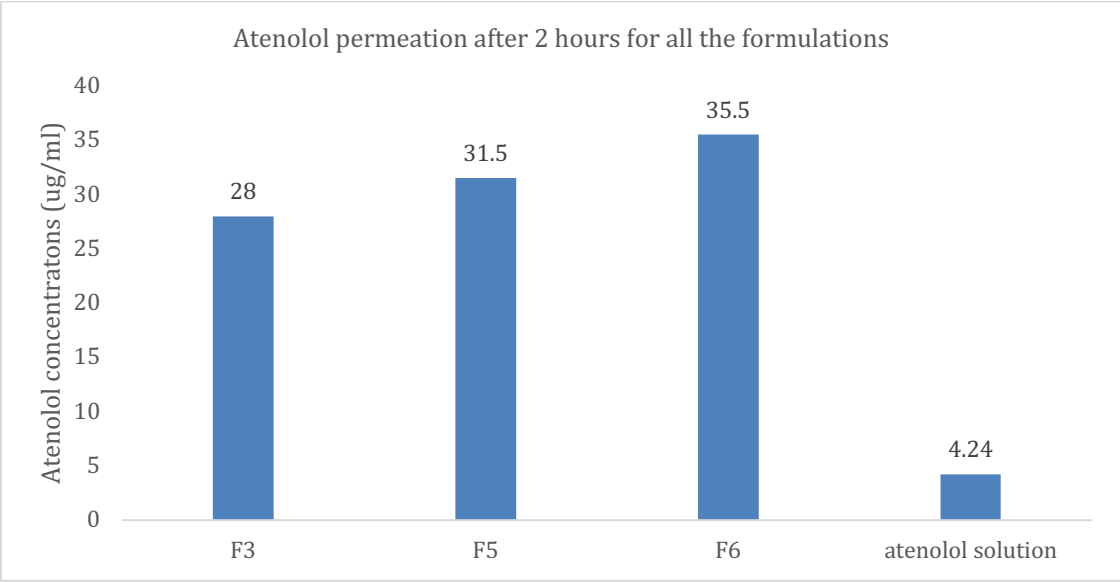

Figure 3.1 Atenolol permeation through cat skin 2 hours after topical application compared to aqueous solution of atenolol.

Similarity Factor f_2

Tables 3.1- 3.5 Show the evaluations of the atenolol gel formulations using the similarity factor 2 (f_2). The tables show the numerical details for how the similarity factor was calculated. Tables 3.1 and 3.2 show the Similarity Factor for F5 to the F6 as a reference product and F3 to F6 respectively. No differences were found between the diffusion of atenolol through cat skin for the test formulations using the similarity factor 2 (51.37 and 50.44) for formulation 5 to 6 and formulation 3 to 6 respectively). This is not surprising as the statistical tests (t-test) performed in the previous chapter along with f_2 evaluation show the discrimination criteria needed to differentiate between topical dosage forms has not fully been delineated and choice of the best formulation is often left to the formulator.

Time	Test (Formulation2)	Reference (Formulation 6)	Reference – Test	$\sum(Rt-Tt)^2$	$\sum(Rt-Tt)*1/n$	$1+\{(Rt-Tt)*1/n\}$
15min	19.78	20.03	0.25	0.0625	17.336	18.336
30 min	37.61	38.14	0.53	0.2809		
2hr	48.53	49.21	0.68	0.4624		
4hr	60.62	61.2	0.58	0.3364		
6hrs	63.34	64.3	0.96	0.9216		
8hrs	68.72	69.4	0.68	0.4624		
12hrs	79.76	76.1	-3.66	13.3956		
24 hrs	83.42	94.5	11.08	122.7664		
			Sum	138.688		

$\{1+(Rt-Tt)*1/n\}-0.5$	$\{1+(Rt-Tt)*1/n\}-0.5*100$	$\log [\{1+(Rt-Tt)*1/n\}-0.5]*100$	$50+\log [\{1+(Rt-Tt)*1/n\}-0.5]$
0.23353254	23.35325405	1.368347404	51.3683474

Table 3.1The similarity factor ((f_2) of formulation 5 to the reference formulation 6 (cat skin).

Time hrs	Test (Formulation 3)	Reference (Formulation6)	Reference – Test	∑(Rt-Tt)2	∑(Rt-Tt)*1/n	1+{(Rt-Tt)*1/n}
0.25	0	20.03	20.03	401.2009	1293.064	1294.06
0.5	9.451	38.14	28.689	823.0587		
2	10.16	49.21	39.04290	1524.348		
4	28.98	61.2	32.21545	1037.835		
6	32.509	64.3	31.79087	1010.659		
8	31.422	69.4	37.9777	1442.305		
12	34.68	76.1	41.41728	1715.391		
24	45.61	94.5	48.88472	2389.716		
			Sum	10344.517		

{1+(Rt-Tt)*1/n}-0.5	{1+(Rt-Tt)*1/n}-0.5*100	log[{1+(Rt-Tt)*1/n}-0.5]*100	50+log [{1+(Rt-Tt)*1/n}-0.5]
0.027798541	2.779854136	0.444022008	50.44402201

Table 3.2 Similarity factor (f_2) of the average concentrations of atenolol transported through cat skin via formulation 3 to the formulation 6 (reference formula).

Tables 3.3 presents the Similarity Factor 2 (f_2) for all the formulations compared to the reference formulation 3 for diffusion through synthetic membrane. Formulation 3 produced the highest atenolol Flux through synthetic membrane and therefore was used as a reference formulation, the results of (f_2) showed similarity between all the formulations.

Synthetic membrane (formulation no) (formulation 3 is the reference)	Similarity factor
Formulation 1	50.39
Formulation2	50.49
Formulation4	50.53
Formulation5	50.59
Formulation6	50.74

Table 3.3 The similarity factors (f_2) of the average concentrations of atenolol transported through synthetic membrane by all formulations to the formulation 3 (highest influx).

Table 3.4 presents the Similarity factor 2 values for each Formulation compared to Formulation 6 as a reference product through cloned human epidermis. F6 produced the highest atenolol diffusion through the cloned human epidermis. The (f_2) results as shown in this table indicates that the formulations F3 and F5 are similar to F6.

Cloned human epidermis (formulation no) F6 is the reference	Similarity factor
Formulation 3	50.6
Formulation 5	50.48

Table 3.4 Similarity factor (f_2) for Formulations 3 and 5 for cloned human epidermis.

Cat skin (formulation no) formulation 6 is the reference product	Similarity factor
Formulation3	50.44
Formulation5	51.36

Table 3.5 Similarity factor (f_2) for formulations 3 and 5 for cat skin.

Table 3.5 summarizes the result of the similarity factor studies of the three selected formulation through cat skin. Formulation 6 produced the highest flux of atenolol through cat skin and used as a reference formulation. (f_2) indicates that no differences between all the formulations.

Dissimilarity (Difference) Factor f_1

Cat skin Formulation 6 is the reference product	Dissimilarity factor f_1
Formulation 3	7
Formulation 5	3

Table 3.6 The dissimilarity factor f_1 of the formulations through cat skin.

The difference factor did not reach 10% for any of the formulation comparisons. Tables 3.6 - 3.8 reveal that maybe one formulation (formulation 1) was different from the other formulations. A borderline value of 10 was determined for the difference factor between formulation 1 to formulation 3 with the synthetic membrane. All other values for the difference factor were well within the acceptable range showing no difference in the permeation of atenolol from the formulation to the other formulations through any of the other membranes (synthetic, cloned human skin or cat skin) studied.

Table 3.7 presents f_1 values for each formulation in cloned human skin where F6 is the reference formulation gave an f_1 value of 8% for F3 to F6 and 7% value for F5 to F6. Table 8 gives the f_1 values for diffusion through synthetic membrane. All f_1 values were under 10% except for F1 compared to F3 at synthetic membrane. Table 3.9 give the analysis of f_1 and f_2 comparing diffusion through cat ear and neck skin. There appears to be no difference in diffusion through ear skin or neck skin.

Cloned human epidermis formulation 6 is the reference.	Dissimilarity factor f_1
Formulation 3	8
Formulation 5	5

Table 3.7 The dissimilarity factor f_1 of the selected formulations through cloned human epidermis.

Synthetic membrane formulation 3 is the reference	Dissimilarity factor
Formulation 1	10
Formulation 2	9
Formulation 4	8
Formulation 5	6
Formulation 6	3

Table 3.8 The dissimilarity factor f_1 of the formulations through synthetic membrane.

Formulation 6	Similarity factor f_2	Dissimilarity factor f_1	t- test
Neck skin	53.9	5.45	0.213
Ear skin profile is the reference.			Not Significantly different.

Table 3.9 The statistical analysis of the similarity and difference factors of the neck and ear skin.

Partial AUC and AUMC

Table 3.10 presents the partial AUC from zero to 24 hours of the atenolol diffusion data through cat skin. It indicates that formulation 3, 5, and 6 are significantly different. The percent difference between F3 and F5 is more than 7% and is more than 7% for F5 to F6 and F3 to F6.

The (pAUC) or (pAUMC) tests as shown in Tables 3.10 – 3.12 indicate a statistically significant improvement for one formulation over any of the others. Formulation 6 did have consistently higher (pAUC) and (pAUMC) values over all other formulations. This occurred with cat ear skin and cloned human skin diffusion data. Also (pAUC) and (AUMC) values for atenolol diffusing through ear was greater than through cat neck skin.

Formulation code	Cat skin Partial AUC 0 to 24	Cat skin Partial AUMC 0 to 24	% difference
Formulation 3	866.76	11907.595	F3 to F6 is more than 7%
Formulation 5	1057.595	14776.47	F5 to F6 is more than 7% (significantly different)
Formulation 6	1318.78	19627.44	All F3, F5 and F6 significantly different.

Table 3.10 The partial AUC of the selected formulations through cat skin (F3, F5 and F6).

Formulation code	Cloned human epidermis Partial AUC 0 to 24	Cloned human epidermis Partial AUMC 0 to 24	% difference
Formulation 3	311.84	6524.09	F3 to F6 is more than 7%
Formulation 5	590.23	12723.582	F5 to F6 is less more 7% (significantly different)
Formulation 6	985.475	20670.205	All F3, F5 and F6 significantly different.

Table 3.11 The partial AUC through cloned human epidermis of the selected formulations (F3, F5 and F6).

Neck versus ear skin comparison studies

Formulation 6	Neck skin	Ear skin
pAUC	1273.61	1339.909
pAUMC	17712.725	19830.3375

Table 3.12 shows the partial AUC for cat neck and ear skin.

The % difference between pAUC for ear skin to neck skin is less than 7% and is more than 7 for the pAUMC % difference.

The Mean Diffusion Time (MDT) was calculated for formulations 3, 5, and 6. Table 3.13 presents the MDT for each formulation diffusion profile. The MDT modestly increases from 13.73 for formulation 3 to 14 for formulation 5 to finally 14.8 for formulation 6. No statistical difference could be found between these values.

Formulation code	MDT Cat skin	MDT Cloned human epidermis
Formulation 3	13.73	20.8
Formulation 5	14	21.5
Formulation 6	14.8	20.9

Table 3.13 The MDT of each formulations.

DISCUSSION

No differences were found between the atenolol in the Similarity Factor (f_2) for F5 to F6 as a reference product and F3 to F6 respectively for the test formulations. This is not surprising as the statistical tests performed in the previous chapter along with f_2 evaluation show the discrimination criteria needed to differentiate between topical dosage forms has not fully been delineated and choice of the best formulation is often left to the formulator. The formulation determined for application to cats for the in vivo study was selected by which formulation produced the greatest atenolol diffusion through cat skin. Formulation 6 not only had the greatest diffusion through cat skin it also provided more consistent release as observed in the atenolol permeation profiles.

The evaluation studies that were carried out to show the discrimination criteria needed to differentiate between the different formulations and in the different barriers (synthetic, cloned epidermis and cat skin) in this study was not helpful to indicate which formulation performed better. The optimal formulation was determined by selecting which formulation that produced the greatest atenolol diffusion through cat skin. Formulation 6 not only had the greatest atenolol diffusion through cat skin it also provided more consistent release as observed in the atenolol permeation profiles.

Only some of the tests indicated a statistically significant improvement for one formulation over the others, formulation 6 did have consistently higher pAUC and AUMC values. This occurred with cat ear skin and cloned human skin diffusion data. Also pAUC and AUMC values for atenolol diffusing through ear was greater than through cat neck skin. No criteria has been set for assessing pAUC and AUMC as a means to determine if significant

differences occur in test studies. It is noteworthy to recognize that guidance by the FDA and their tacit approval for criteria to determine differences (f_1) or similarity (f_2) between two formulations did not provide direction in the selection of which formulation to use in the in-vivo study.

The Dissimilarity Factor (f_1) is designed to calculate the present difference between two diffusion curves at each time point. In addition, it is a measurement tool of the relative error between two curves. According to table 3.6, the three selected formulations (F3, F5, and F6 through cat skin) are not significantly different ass the f_1 values stayed with 10% indicating similarity between all formulations.

Values of f_1 for each of the formulations using F6 as a reference standard through cloned human skin, and Cat skin had values under 10%. F1 compared to F3 in synthetic membrane produced a 10 % but all other f_1 values were under 10%. The values of similarity and dissimilarity appear inadequate to differentiate between the diffusion profiles of all formulations through the membranes used in the study.

Calculation the Mean Diffusion Time (MDT) from statistical moments is a comparison technique that has been used as a tool to describe different in vitro dissolution, and diffusion profiles, curves of different shapes and extent that cannot be differentiated by other means but have a minimum of errors. The higher MDT indicates slow release of drug molecules for the formulation. The MDT values are very close in value and t-tests indicated that they are no statistically different.

The pAUC values of the cat neck compared to ear skin shows the close similarity of the pAUC and pAUMC values.

All diffusion profiles through cat neck and ear skin are similar based on $f_1$and f_2. The total amount to atenolol diffusing through cat ear skin is more than through neck skin probably due the thickness of the cat ear skin is slightly thinner that the neck skin. Applying a gel to the ear is preferable due the fact no shaving of the fur is required as is in the case of application to the neck area.

The effect of enhancers on atenolol through cat was significant. The aid the percutaneous enhancers had on atenolol diffusion through cat was compared to a 1% atenolol solution. Formulations 3, 5 and 6 increased atenolol penetration through cat skin by 6.8, 7.4 and 8.3 times greater than the atenolol solution alone. The solubility of atenolol in 26.5 mg/ml in water. The solubility of atenolol was determined to be slightly greater than 100 mg/ml in the co-solvent system of formulation 6, four times increase in solubility in water. The increase in solubility of atenolol in the co-solvent does not nearly account for the increase in the increase in permeation of atenolol through cat. The use of penetration enhancers was highly effective and necessary to produce sufficient atenolol penetration through cat skin to achieve therapeutic concentrations.

The applicability of these methods for comparing diffusion profiles is quite limited in their ability to differentiate between curves, especially for zero-order diffusion curves. It becomes clear why the FDA has difficulty assessing interchangeability of topical products that are commercially sold.

CONCLUSION

Three different atenolol formulations were developed in order to compare their performance as potential vehicles for achieving effective skin delivery of atenolol that would achieve system therapeutic concentrations. The best formulations selected were tested to evaluate their ability to deliver the drug into the cat skin in comparison to a simple aqueous atenolol solution containing the same amount of drug (1% w/v). All the atenolol formulations markedly ($p < 0.001$) improved the amount of drug that penetrated through the skin layers compared to the simple aqueous solution. A minimum of a nearly 700% increase atenolol penetration through cat skin in the case of Formulation 3, to 750% for Formulation 5 and up to a maximum of 900%, in the case of Formulation 6 was observed. In particular, Formulation 6 is containing DMI 15% as drug carrier showed greater diffusion of atenolol though cloned human skin and cat skin and provided better consistency terms of drug delivery. It also gave rise to the highest increase in drug penetration ability through the skin, probably due to the simultaneous presence in their composition of ethanol and polysorbate 80, propylene glycol, glycerol and 15 % DMI all acting as permeation enhancers**.**

The results of the similarity factor tests on the three selected formulations through cat skin revealed Formulation 6 produced the highest flux of atenolol through cat skin and was used as the reference formulation. (f_2) indicated that no differences were detected between all the formulations. The evaluation studies that were carried out to show the discrimination criteria needed to differentiate between the different formulations and in the different barriers (synthetic, cloned epidermis and cat skin) in this study was not helpful to indicate

which formulation is the optimized one. However, Formulation 6 did have consistently higher pAUC and AUMC values. This occurred with cat ear skin and cloned human skin diffusion data. In such cases the choice of the best formulation is often left to the formulator. The formulation was determined by selecting which formulation produced the greatest atenolol diffusion through cat skin. Formulation 6 is not only had the greatest diffusion through cat skin it also provided more consistent release as observed in the atenolol permeation profiles. The inability of the evaluation tests to clearly reveal equivalence between topical products creates a difficult situation for the FDA assessing interchangeability of topical products that are commercially sold.

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CHAPTER 4

INVIVO TRANSDERMAL DRUG DELIVERY OF ATENOLOL

: In vivo pilot study

Sumeia M. Mohamed

ABSTRACT

Atenolol is a β1-receptor antagonist commonly prescribed in cats affected with hypertrophic obstructive cardiomyopathy. Veterinarians are trained to easily administer medication to animals, whether it is by pill, injection or IV. However, clients/owners sometimes face difficulties administering oral medication at home, oftentimes feeling anxiety about how they can get their pet to swallow a pill. The main goal of this project is to achieve successful transdermal delivery of atenolol to cats. Based on a previous study that investigated transdermal administration of atenolol that reported sub-therapeutic serum atenolol concentrations after topical application, the present study's main object is to administer an optimized transdermal atenolol formulation to 11 healthy cats (mixed breed). The optimized formulation (developed in the in-vitro studies chapter) that contained 15% DMI (dimethyl-isosorbide) was chosen for application to cats. Each cat received an escalating dose of transdermal atenolol over 10 days. On day 10 after initiation of transdermal atenolol administration; blood samples were collected to measure atenolol concentrations at selected time points (3, 6, 12 hours) after transdermal atenolol application. Also, after phlebotomy was performed at the time points (3, 6, 12 hours) the cats were followed up with an ECG and average heart rate (HR) measurement. Gradual tapering of transdermal atenolol subsequently occurred over 4 days. Results: All enrolled cats successfully completed the clinical trial and the results are as following: no adverse side effects were reported in any cat. Due to formulation drug stability issues (a single batch of transdermal gel) four cats were excluded from the study results. Six of 7 cats

achieved therapeutic serum atenolol levels for at least one time point. Specifically, 4 of 7 cats had therapeutic serum atenolol concentrations 3 hours post-atenolol. At 6 hours post-atenolol time point, only 1 had a therapeutic serum atenolol concentration. At 12 hours post-atenolol dosing, 4 of 7 cats had therapeutic serum atenolol concentrations. For the HR data post-phlebotomy, there was moderate correlation ($r = 0.66$) between absolute HR and serum atenolol concentrations. The specific formulation used for transdermal atenolol administration at 25 mg q12h resulted in attendant HR reduction in clinically healthy cats and therapeutic serum atenolol concentrations in the majority of cats. This preliminary data requires more investigation and validation in a larger number of cats.

INTRODUCTION

Cats with cardiac arrhythmia are treated with atenolol in order to adjust heart rate and reduce blood pressure. In veterinarian medicine and especially in cats, atenolol tablets are used to treat hypertrophic cardiomyopathy (HCM), left ventricular outflow tract obstruction and hypertension secondary to hyperthyroidism (Jackson BL et al., 2015). The direct specific cause of how cats develop hypertrophic cardiomyopathy (HCM) is unknown; but the presence of some factors makes the disease more likely to happen. One factor is the dietary deficiency of taurine; an essential amino acid. Many cat canned food lacks this required supplement (Ripps. H and Shen W, 2012). The association of the disease with taurine deficiency caused the addition of taurine to cat's food to make sure they have a sufficient amount. Also tuna or fish based food are more likely to facilitate hyperthyroidism which leads to overload on the cat's heart, thickening of the heart muscle and development of enlarged heart in cats (Liu .SK et al., 1984). Other chemicals in canned foods can be factors that might contribute to heart disease. There is an association between the modern home environment and cats developing heart disease. In some feline families there is a genetic predisposition for this condition (cardiomyopathy) like Maine coon cats. But generally, the association cannot be proven, although heart disease has been documented to have higher incidence rate in American short hair and Persian cats (Meurs

K, 2016). Heart disease most commonly occurs in cats between ages of 5 to 7 years, but recorded cases have ranged from 3 months to 17 years. Incidence rates of this disease is higher in males. (https://www.manhattancats.com/article-archive/cardiology/hypertrophic-cardiomyopathy)

Atenolol is superior to diltiazem as it has shown a greater ability to reduce the heart rate and consequently reduce left ventricular obstruction (Weir MR et al., 1987). Cats with heart disease are given an atenolol dose of 6.25-12 mg twice/day, usually tablets or pills. Administration of oral tablets to cats can be a challenging issue for owners. Often owners' unwillingness to administer oral tablets to cats results in subtherapeutic atenolol concentrations in cats. Therefore, there is an increasing demand to develop an alternative route of administration for atenolol to cats.

In human, billions of transdermal drug dosage forms (creams, ointments, pastes, gels, transdermal patches, etc.) for several drugs are produced every year. Thus, clinical experience suggests use of transdermal drug formulations to administer atenolol for cats may be beneficial. Although, the bioavailability of atenolol is 90 +/- 9% after oral administration (Quinones M et al., 1996) a transdermal preparation of atenolol is considered desirable.

It is well known that the highly effective barrier properties of the Stratum Corneum significantly limits transdermal delivery of drugs. Among the ways to overcome Stratum Corneum limiting of drug permeation is drug modulation within the Stratum Corneum. There are a number of mechanisms that produce a temporary impairment of the Stratum Corneum barrier function. An important approach to modulation of the penetration of drugs through the stratum corneum after a topical application is the use of chemical penetration enhancement.

The mechanisms of action of penetration enhancers are very complex and not yet fully understood. Basically, two main pathways exist in the stratum corneum for drug transportation through the intercellular lipid matrix, namely hydrophilic lipids and lipophilic lipids pathway (Trommer and Neubert, 2006). Enhancers work by arrangement of polar head groups of the Stratum Corneum lipids (known as the hydrophilic pathway) to promote hydrophilic drug penetration. The second pathway facilitates the lipophilic drug penetration and known as lipophilic pathway. Enhancers work on molecular organization of lipid's hydrocarbon chain. However, the enhancers that affect the hydrophilic pathway also play role in the ordering of the hydrophobic tails of the Stratum corneum lipids and vice versa. This property explains the improvement of the penetration of lipophilic drugs when using hydrophilic enhancer and the opposite when using lipophilic enhancer for the hydrophilic drug (Magnusson et al., 2001).

The published literature on transdermal atenolol administration to cats in veterinary medicine is limited. One group evaluated the pharmacokinetics and pharmacodynamics

variables of a single atenolol dose given intravenously and orally to cats. At 6 and 12 hours post administration significant decreases in heart rate were noted following isoproterenol challenge in all cats with a minimum atenolol plasma concentration at more than 260 ng/ml (Quinones M et al., 1996). Another small study compared atenolol pharmacodynamics after oral and transdermal administration in healthy male cats. This group found cats receiving oral atenolol reached therapeutic concentrations after oral administration, whereas cats receiving the transdermal formulation had atenolol concentrations below 260 ng/ml at the same time point (Macgregor JM et al., 2008). The study reported that two cats out of seven attained a therapeutic atenolol concentration with average peak plasma concentrations for all cats of 173+/_122 ng/ml and mean trough concentrations of 62.4+/_17 ng/ml which produced a negative correlation between plasma atenolol concentrations and the cat's heart rate.

Due to the lack of success by previous investigators in achieving therapeutic atenolol concentrations in cat after topical administration it was felt that an improved topical formulation could provide a better platform to treat cats with a topical atenolol dosage form. This study was initiated to examine the possibility that a better designed topical formulation of atenolol will provide an improved therapeutic response.

METHODS AND PROCEDURES AND MATERIALS

Only adult healthy cats presented to the OSU Cardiology service were included in the in-vivo study. All study participants underwent a physical examination, blood pressure measurement, electrocardiogram, and echocardiography. To ensure appropriate renal and hepatic function, baseline blood work was also performed. Informed consent was obtained by all owners, and the study was approved by the Oregon State University and College of Veterinary Medicine's Institutional Animal Care and Use Committee. Cats showing normal screening values were considered healthy and included in the study.

While previous studies have used a lead-in period of 7 days prior to sampling, a longer duration of administration may actually result in higher plasma concentrations due to better disruption and softening of the stratum corneum (Macgregor et al., 2008). In this study the enrolled cats received concentrated transdermal atenolol (12.5 mg/0.1mL) on the inner pinna of ears, alternating the application of the atenolol gel to ears. Application of atenolol went according to the following schedule: 12.5 mg once daily for 2 days, then 12.5 mg every 12 hours for 2 days, then 25 mg in the morning followed by 12.5 mg 12 hours later, then 25 mg every 12 hours for 3 days. On the 10th day of treatment, 25 mg of transdermal atenolol was applied in the morning. Then cats were admitted to the small animal veterinary hospital at OSU for participation in the study. Blood samples for measurement of serum atenolol concentration were collected at 3, 6, and 12 hours after drug administration. Blood samples were analyzed by an outside laboratory in Oklahoma by HPLC ms/ms.

Additionally, a 7-lead ECG was performed at the 3, and 6 hour time points right after phlebotomy and an average heart rate (HR) was measured from a 30 second recording. Atenolol was not administered to any cat in the evening of hospitalization with a tapering dose schedule consisting of 25 mg once daily for one more day, followed by 12.5 mg once daily for 2 days, then the treatment was discontinued.

Transdermal atenolol gels were packaged in syringes to make it easy to administer and dispense proper dosages. The animal's dose was put into the smallest amount of gel, 0.1ml containing 12.5 mg. The transdermal gel was applied to the animal's inner pinna. The clients were advised to wear gloves or finger cots and apply each dose to the alternate ear.

Pharmacokinetics analysis

A simulation convolution approach for a pharmacokinetic model utilizing an initial burst flux (40 % of dose) of atenolol for administration (39.5 µg over 3 hours) followed by a zero-order flux (2.7µg/hours) of atenolol across cat skin beginning 3 hours after atenolol topical application. Elimination of atenolol was assumed to be by a first-order process. Pharmacokinetic parameters were developed by closeness of fit of simulated data to actual data (WinNonLin; Certara USA, Inc., 100 Overlook Center, Suite 101, Princeton, NJ 08540 USA).

Simulation Convolution Pharmacokinetic Model

$C(t) = f_1(C_1 t) + f_2(C_2 t) - f_3 C_{1t}$.

Where $f_1(C_1 t)$ is the burst effect absorption function.

$f_2(C_2 t)$ is the zero-order sustained release absorption function.

$f_3(C_1 t)$ is a first order elimination process function.

Estimation of the pharmacokinetic variables of transdermal atenolol was done to produce the half-life, clearance, volume of distribution and the elimination rate constant. ($T_{1/2}$, k_{el}, Cl= 259 ml/min and V_d).

Statistical Analysis of Atenolol Pharmacodynamics

Based on the small sample size, the data was considered nonparametric. The relationship between absolute HR and plasma atenolol concentration was evaluated using linear modeling by Spearman's rank correlation coefficient. The difference in baseline HR to stress provocation was assessed using the Wilcoxon matched-pairs signed rank test for each cat. Values of $p < 0.05$ were considered significant.

RESULTS

For the in-vivo topical atenolol administration second phase of the study, 17 cats underwent the aforementioned screening, only 11 cats met inclusion criteria. Exclusion from being in the study was due to several different reasons such as renal dysfunction (n=2), frequent ventricular ectopy (n=1), equivocal/mild structural heart disease (n=3). All enrolled cats successfully completed the clinical trial and no adverse side effects were reported. At the beginning of this part of the study; two cats received a maximum transdermal dose of 12.5 mg q12h (following the predesigned study steps) and it was reported that serum atenolol concentrations were less than the minimum therapeutic level 260 ng/mL at 3, 6, and 12 hours post-atenolol administration. The topical dose administered was likely sub-therapeutic for this transdermal formulation of atenolol hence this data was not included in the final data analysis and the atenolol dose was increased for future subjects in the study. The transdermal atenolol dose was increased to 25 mg q12h, and the same 2 cats were re-enrolled in the study and given double the atenolol dosage after a 4 weeks washout period from the first topical atenolol administration. All subsequent cats enrolled in the study received the higher transdermal atenolol dose of 25 mg q12h. All formulations were tested for atenolol concentration prior to dispensing with a target of 12.5 mg/0.1 ml ±10% of the intended concentration.

Formulation 6 was tested in terms of stability and compatibility. The results showed no significant degradation in atenolol concentration over a 180-day period. A second lot of the formulation showed discoloration revealing atenolol degradation within 28 days. When it was applied to four cats, atenolol concentrations did not reach therapeutic levels. A

stability study as detailed in chapter 5 was initiated. The data of the four cats was not included in the final results of the study due to atenolol instability.

For the remaining 7 cats, 6 cats had therapeutic serum atenolol concentrations at least one time point. Specifically, 4 of the 7 cats had therapeutic serum atenolol concentrations 3 hours post-atenolol and 1 additional cat had a near therapeutic concentration of 255 ng/mL (target ≥ 260 ng/mL) at 3 hours post-atenolol. At 6 hours post-atenolol, only 1 of the 7 cats had a therapeutic serum atenolol concentration and another 3 cats had near therapeutic serum atenolol concentrations. At 12 hours post-atenolol dosing, 4 of the 7 cats had therapeutic serum atenolol concentrations (Figure 4.1).

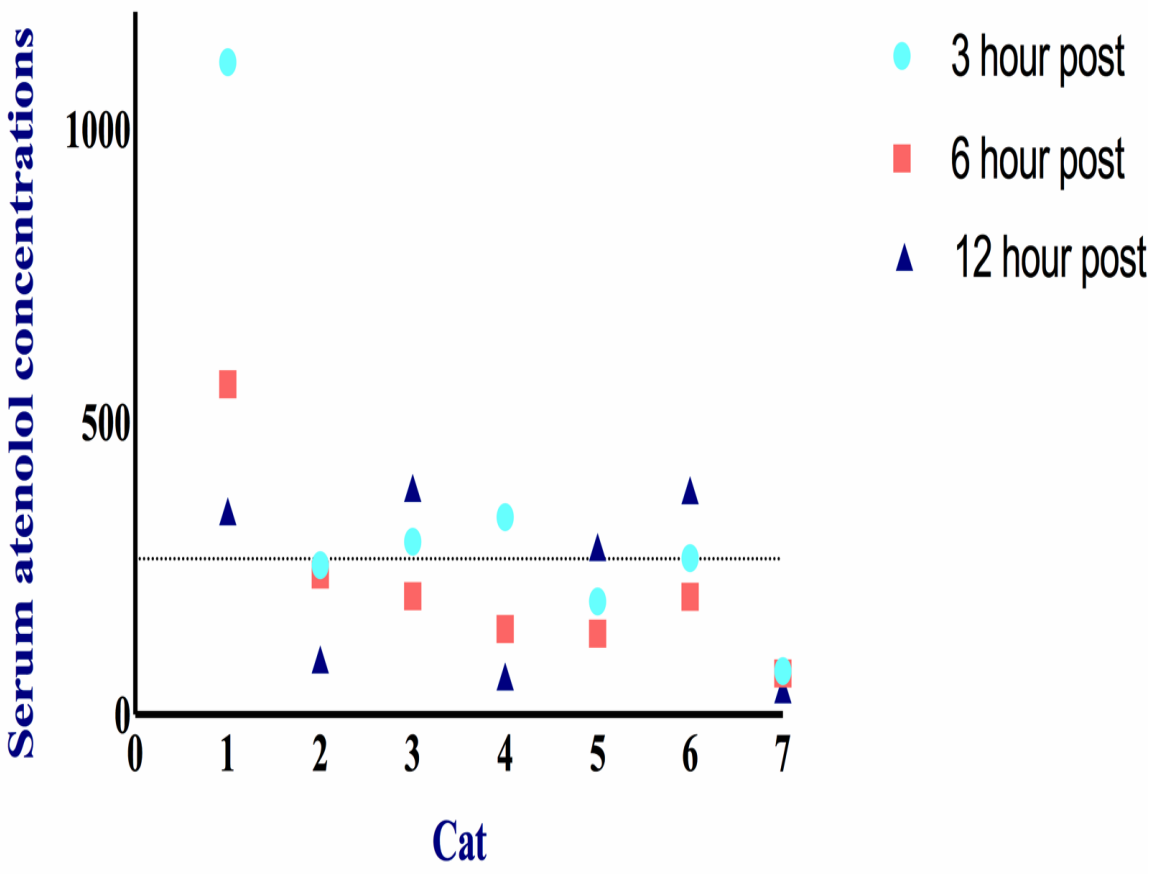

Figure 4.1 Serum Concentrations of atenolol in different time points for each of the cats.

Figure (4.1) shows the individual time point concentrations of atenolol after transdermal application. The results suggest that transdermal atenolol formulation was able to deliver the desired amount of atenolol to cats producing a therapeutic effect. As shown above the transdermal formulation provided therapeutic atenolol levels of atenolol up to 12 hours. A sustained release of atenolol from the transdermal preparation was attained by this optimized formula. Generally, veterinary practitioners prefer to reduce the number of the required doses given to pets by selecting sustained release preparations due to the difficulties that accompany pet dosing.

Cat Name	Sex	HR baseline average	HR 3 hrs post-atenolol	HR 6 hrs post-atenolol	HR 12 hrs post-atenolol	Age of the cat (years)
Winter Elmore	SF	180	140	170		4.5
Lydia Hoff	SF	200	160	180		7.5
Beatrice Treseder	SF	280	180	-		9
Bulleh Forman	MC	220	180	190		3
Leeloo Herdener	MC	160	180	180		2.75
Maximus MacClenathan	MC	200	180	200		1.75
Bang Bang Denninger	SF	160	140	160		6
Crème Steigerwald	MC	180	140	160		6.5
Pippin Webb	MC	220	180	180	180	1.3
Sophie Sirochman	SF	160	140	140	120	2.25
Simone Ihrig	SF	180	180	180	180	8.5

Table 4.1 The heart rate base line obtained prior to application daily topical atenolol dose for the clinical trial cats and the reported reduction in the heart rate post atenolol transdermal application.

Table 4.1 presents the results of the pharmacodynamic studies that carried out on the enrolled cat post atenolol formulation application. The table shows the HR base line for each cat prior to transdermal atenolol administration and the results of a significant reduction in the heart rate post atenolol application.

For the HR data, the median baseline HR was 200 bpm [IQR 180-220). At 3 hours post-atenolol, the median HR was 180 bpm [IQR 140-180] and at 6 hours post-atenolol administration was 180 bpm [163-183], Table 4.1. There was moderate correlation (r=0.66) between absolute HR and serum atenolol concentration. There was a statistically significant reduction in HR 3 hours post-atenolol (p=0.03) and no statistical difference in HR 6 hours post-atenolol (p=0.06).

A simulation of the atenolol cat serum concentrations versus time profile was generated and compared to the actual cat atenolol serum concentration over time. Using a burst effect for absorption of atenolol for the first 3 hours that amounted to 40 percent of the content of atenolol in the dose administered followed by a zero-order absorption process gave atenolol concentration time curve similar to actual atenolol cat concentrations time profile. The sustained zero-order absorption rate was 2.7 ug/cm^2/hr (Figure 4.2). This provided a method to estimate pharmacokinetic parameters for half-life, K_{el}, clearance and volume of distribution. This was done using WinNonLin to fit the simulated data to the empirical date. The values for Half-life was 3.44 hr +/- 0.5, K_{el} = 0.2 hr^{-1}, Cl = 259 +/- 72 ml/h/kg and V_d = 1,088 +/- 148 ml/kg were obtained.

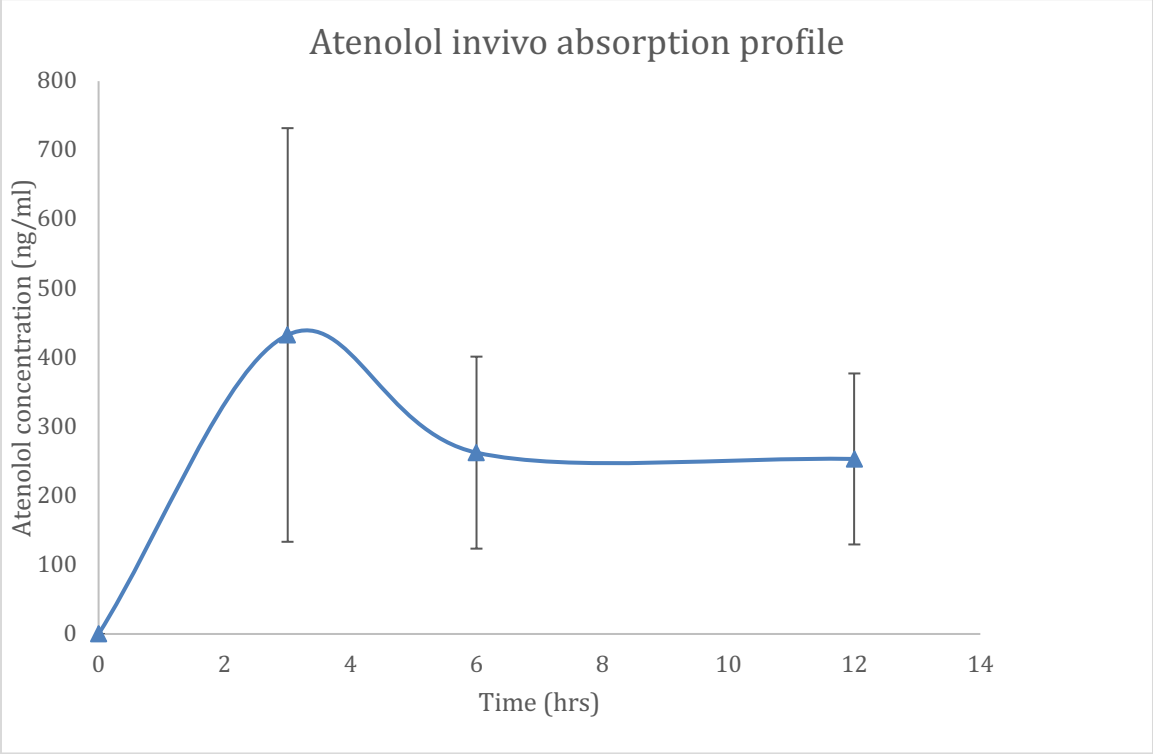

Figure 4.2 The average atenolol plasma concentrations in cats after atenolol gel application in the clinical trial study.

Table 4.2 along with Figure 4.2 give the actual plasma concentrations of atenolol in cats after topical administration. Table 4.3 gives the predicted concentrations of atenolol in cat from topical administration if the fluxes obtained in the in vitro study were applied to the pharmacokinetic model. There was a 26% increase in the actual peak atenolol concentration in cats over the predicted. Other concentration values for atenolol at 6 and 12 hours were closer to the observed values.

Time hours after atenolol administration (transdermal)	Average atenolol concentration (all the cats) ng/ml
0	0
3	432.714286
6	262.428571
12	253.285714

Table 4.2 The average atenolol concentrations in cat plasma post administration of transdermal atenolol gel.

Time (hours)	Average atenolol concentration (simulating values)
0	0
3	363.708
6	238.7
12	219.4

Table 4.3 The simulating atenolol concentration from the pharmacokinetic model using the fluxes observed in the in vitro tests (*invitro* studies).

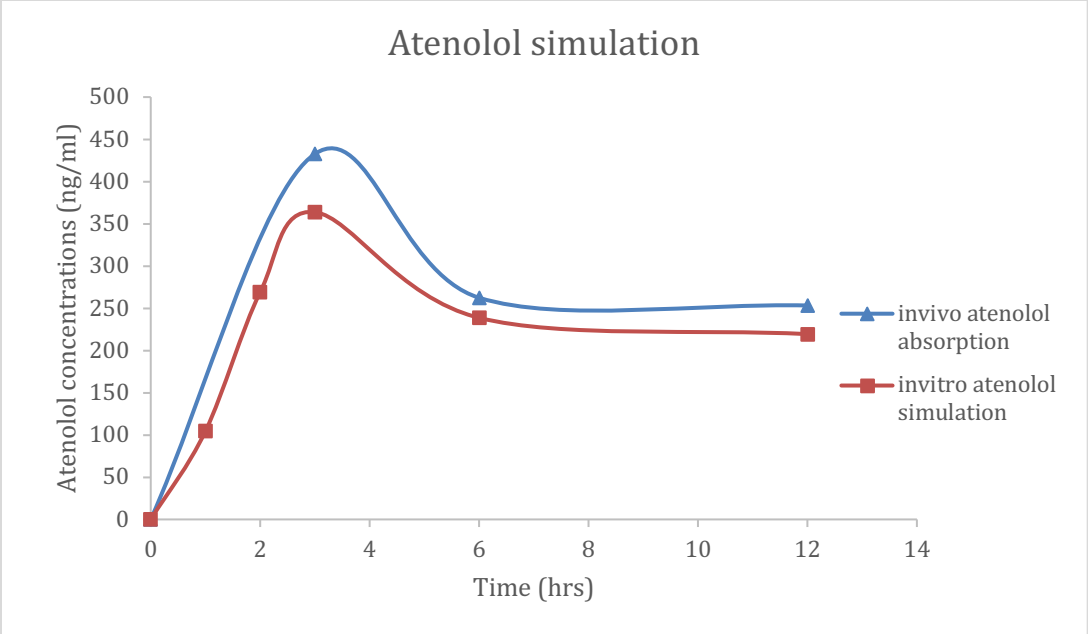

Figure 4.3 Comparison of Actual Atenolol serum concentrations to simulated cat serum concentrations where a burst flux rate of atenolol absorption followed by a zero-order flux rate of absorption.

Figure 4.3 shows the correlation ability of the pharmacokinetic model to predict plasma concentrations using in vitro fluxes and published pharmacokinetic parameters to yield accurate results for comparison for the selected atenolol formulation.

DISCUSSION

The previous chapter of this thesis mentions in detail six formulations. All formulations have the same concentration of the active ingredient (Atenolol). According to veterinary medicine law, all constituents of topical formulations were commercially available. Previous results for Formulations 3, 5, and 6 exhibited the greatest permeability through synthetic membrane, cloned human epidermis and cat skin mounted on Franz-Chin diffusion cells. As a consequence, these three formulations were selected for further evaluation in cat skin. Also the site of application was investigated to determine the most permeable area of cat skin to atenolol from these 3 formulations. The formulations were applied to the excised pinna and nape skin samples. The results showed a greater permeability was across the pinnae than neck skin. Considering the *in-vitro* results, formulation 6 provided greater permeation of atenolol through cat skin and was selected as the optimized formulation for *in-vivo* transdermal application to cats to test atenolol efficacy in reducing heart rate.

The results of this study suggest a dose of 25 mg per cat q12h provided by the optimal formulation produced therapeutic serum atenolol concentrations in the majority of cats with attendant HR reduction. Transdermal administration of medications has desired advantages compared to oral and intravenous medications especially in vet medicine. Therapeutic concentrations at 12 hours post administration of atenolol is encouraging. The explanation of the prolonged therapeutic atenolol concentrations at 12 hours after topical administration is that the burst effect of drug absorption through the skin had declined and the zero-order absorption process of atenolol drug diffusion across the skin membrane had time to build

to where more drug can be absorbed into the systemic system through the skin sufficiently providing sustained release to achieve therapeutic concentrations from transdermal atenolol. The atenolol concentration at the 6 hour time points were sub therapeutic most likely due to the initial burst of topical absorption of atenolol had worn off and the second sustained topical delivery of atenolol had not quite reached therapeutic atenolol concentrations.

There is a large number of chemical substances that promote a greater drug penetration through the skin. These substances are known as penetration enhancers. Such enhancers include water, alcohols (e.g. ethanol), glycols (e.g. propylene glycol), sulfoxides (e.g. dimethyl-sulfoxide), azone and its derivatives, urea and its derivatives, terpenes and terpenoids (e.g. d-limonene), pyrrolidones (e.g. N-methyl-2-pyrrolidone), cyclodextrins, surfactants (e.g. sodium dodecyl sulfate), fatty acids (e.g. oleic acid) and others (Williams and Barry, 2004). The enhancers chosen in this study (ethanol, DMI, polysorbate 80, glycerin and propylene glycol greatly increased atenolol diffusion through cat skin.

Examining previous studies applying topical transdermal atenolol to healthy cats only a small minority of cats developed therapeutic serum atenolol concentrations (Macgregor JM et al., 2008). Due to the different characteristics of this current formulation as well as an improved study design achieved better results. Considering the information from the previous study it was felt the addition of dimethyl isosorbide and ethanol to the formulation, which are penetration enhancers, as well as using a different surfactant (polysorbate 80) would help promote better transdermal penetration of atenolol. Also waiting a slightly longer time (2 hours) until sampling also would allow for more hydration

of the stratum corneum layer of the epidermis, the main physical barrier to the drug penetration (Riviere JE and Papich MG, 2001) would aid in atenolol dermal penetration.

The major increase in the atenolol dose and dosage regimen that was used in this study was still within the safety margin of the dose of atenolol. The dose used in this study was increased to 25 mg q12h compared to 6.25 mg q12h as that it was felt it would yield a greater possibility of achieving therapeutic atenolol concentration. The minimum concentration cut-off for therapeutic serum atenolol concentration was kept the same (260 ng/mL). Previous researchers performed an evaluation of the beta-blocking effects of oral atenolol using isoproterenol challenge in cats. They reported that the lowest atenolol concentration with adequate β-blockade was 260 ng/mL, while serum atenolol concentrations of 42 ng/mL did not result in adequate β-blockade. The true therapeutic serum concentration cut-off lies between 42 and 260 ng/ml (Macgregor JM., 2008). Examining the effective therapeutic concentration of serum atenolol in human that produces β-blockade is reported to be a concentration from 200-500 ng/mL (Frishman WH, 1980). Based on this information using a cut-off of 200 ng/ml it could be consequently concluded that 6 of 7 cats would have at least one therapeutic time point. Four of 7 cats at 6 hours point and 6 of 7 cats at 12 hours post atenolol topical administration achieved therapeutic concentration. While five of seven cats would be therapeutic at the 3 hours point.

The pharmacokinetic model predicted atenolol concentrations adequately. The curve that was produced by the pharmacokinetic model mirrored the actual plasma concentrations. The fitted pharmacokinetic parameters in the model were close to literature values for

atenolol in cats. This adds a level of predictability that reinforces the assessment that topical administration of atenolol can be effective in treating HCM in cats.

There are issues in this study that should be considered such as topical dosage adjustment from oral dosages and formulation stability. The first part of this *in-vivo* phase, the initial dose of 12.5 mg per cat q12h failed to provide therapeutic serum atenolol concentrations. However, this is not a new finding. The use of a transdermal formulation as direct substitution for oral dosages has been previously documented to provide less consistent and lower plasma concentrations (Hoffman SB et al., 2002). It is well known that the transdermal formulations provide safe and desired advantages regarding the avoidance of the hepatic first-pass metabolism and easier administration than oral medications in cats. The challenges associated with topical delivery (i.e. dosage, formulation, enhancers, etc.) still needs to be addressed.

A number of limitations can be identified in this study. The small number of subjects (healthy cats) in the study. Therefore, the data should be verified in a larger population of healthy and HCM affected cats. It is uncertain if the results can be extrapolated to cats with structural heart disease. Furthermore, the home environment where the application of the transdermal medications takes place can be a source of owner and patient compliance issues. Another limitation is the sampled serum atenolol levels of stressed cats in the hospital setting, which theoretically can lead to changes in blood pressure that also may cause drug accumulation with vasoconstriction through reduced dermal perfusion, leading to drug accumulation in the epidermis but not systemically (Riviere JE and Papich MG, 2001)

CONCLUSION

The first group of cats in this clinical trial were administered a dose of 12.5 mg per cat q12h and failed to provide therapeutic serum atenolol concentrations. However, this was expected but lower dose of atenolol was intentionally used to start the study with. The use of a transdermal formulation as direct substitution for oral dosages has been previously documented to provide less consistent and lower plasma concentrations (Hoffman SB et al., 2002). In order to get a therapeutic level of a drug by transdermal route the dose should be higher than that used for oral dose. It is also well known that the transdermal formulations provide safe and desired advantages regarding the avoidance of the hepatic first-pass metabolism and easier administration than oral medications in cats.

The major increase in the atenolol dose to 25 mg per cat and the dosage regimen (q 12h) that was used in this study was still within the safety margin of the dose of atenolol. The dose used in this study was increased to 25 mg q12h yielded a greater atenolol concentration and was therapeutically sufficient. According to (Macgregor JM et al., 2008) the beta-blocking effects of oral atenolol using isoproterenol challenge starts at the 260 ng/mL, while serum atenolol concentrations of 42 ng/mL did not result in adequate β-blockade. The true therapeutic serum concentration cut-off lies between 42 and 260 ng/ml. The effective therapeutic concentration of serum atenolol in human that produces β-blockade (200-500 ng/mL) (Delima et al., 1995). Using a 200ng/ml lower limit for the therapeutic concentration it could be concluded that 4 of 7 cats would have at least one therapeutic at 6hr time point and 6 of 7 at 12 hours post atenolol topical administration achieved therapeutic concentration. At the 3 hours time points 5 of 7 cats would be

therapeutic. The results of this study suggest the optimal formulation of atenolol administered transdermally at 25 mg q12h provides therapeutic serum atenolol concentrations and attendant HR reduction in most clinically healthy cats. The constituents of the formulation are commercially available for use by compounding pharmacies.

The formulation's atenolol content and stability should be measured by pharmacists prior to dispensing this compounded medication. This preliminary data requires validation in a larger cohort of cats.

In vitro atenolol diffusion was well correlated with *in vivo* absorption. This correlation is valued in modification or development of formulations and prediction of *in vivo* absorption profile.

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CHAPTER 5

ATENOLOL GEL STABILITY STUDIES

Sumeia M Mohamed

ABSTRACT

Stability studies were carried out on atenolol gel containing 12.5 mg/0.1 ml. No significant changes were observed including amount of atenolol in the gel after 6 months storage at room temperature or at 37^{o}C. A second stability study to determine the effect of exposure to light and pH was performed. No differences in atenolol concentration or color was observed between the gel samples stored in a light or dark environment at room temperature for 90 days. The effect pH had on atenolol concentrations was dramatic. When the pH of the stored gel was above 7.5 a drop of 22% in atenolol concentration in the gel occurred in the first 28 days of storage and a 31 % drop in concentration at 90 days of storage. The gel appearance turned to a light brown color when stored at a pH above 7.5. Storage of the gel containing atenolol concentration of 12.5 mg/0.1 ml remained stable without color change at pH values of 6.5, 7.0, and 7.4 for entire time of the stability studies. Gels of Atenolol can remain stable for up to six months when formulated at a pH below 7.4 even at very high concentrations of atenolol (12.5 mg/0.1 ml).

INTRODUCTION

Stability is an essential factor for quality, efficacy and safety of drug products. A drug product with insufficient stability can result in change of their physical as well as chemical characteristics. Gels are semi solid dosage forms used mainly for transdermal drug delivery. Gels have a higher aqueous component that permits easy migration of the drug through a vehicle once the drug is dissolved as it is essentially a liquid compared with ointments or creams (Kumar L and Verma R, 2010). A stability study on an atenolol gel formulation designed for transdermal application that was comprised with ingredients such as Aqupec HV 505, propylene glycol and ethyl acetate was performed. The atenolol content of the gel did not significantly change over 56 days of storage (Chaerunisaa A et al., 2019). Another stability study was performed (Ramachandra M and Ritesh S, 2017) on atenolol transdermal preparations with different concentrations of ethanol and propylene glycol. The atenolol content started at 94.71% ± 1.41and after 30 days the atenolol content was 93.68% ± 1.25. There were no physical changes in appearance, flexibility, color and physicochemical evaluation parameter was slightly changed. The photosensitivity of atenolol to UVA/UVB light was investigated and found to increase as pH values decreased (Andrisano V et al., 1999). This study was initiated to determine if exposure to light or pH has a significant effect on the stability of atenolol formulated in a gel intended for topical treatment of cats.

MATERIALS AND METHODS

The optimized formulation used in the stability study had the following formula (6).

12.5 mg/0.1 ml atenolol

5% Polysorbate 80

15% Propylene glycol

15% Glycerol

10% Ethanol

15% DMI

0.75% Carbomer (934)

0.45% Triethanolamine.

Water qs to 100%

Stability studies

A number of 1 ml syringes from syringes that were prepared for the topical administration study in cats were collected and separated for testing for atenolol stability. The drug stability test was conducted by storing the atenolol gel at different temperature conditions; incubator temperature (37 ±1°C) and room temperature (25 ± 2°C). The 1 ml syringes of

atenolol gel formulations were stored in Parafilm-sealed glass vials. The samples were withdrawn at different time intervals over a period of 6 months and atenolol concentrations in the formulations were analyzed by HPLC. The first stability study was conducted on the three selected formulations F3, F5 and F6.

Discoloration of the transdermal gel was noted with the second batch of the optimized formulation (6) of atenolol gel within a month of gel generation. The second stability study was performed to determine whether storage in light or different pHs caused the instability. The pH of the optimized formulation was adjusted with triethanolamine to give pH's that ranged from 6.5 to 7.8 with an atenolol concentration held at 12.5 mg/0.1 ml. One group of syringes with varying pH were stored in light at room temperature versus an identical group of syringes that were stored in the dark at room temperature for 90 days.

The final atenolol stability study was conducted to compare a Compounding Pharmacy veterinary medicine formulation of atenolol prepared in the Pluronic Organo gel formula (PLO Gel; PCCA) to the optimal formulation (6) used in the study to treat cats. The atenolol concentrations and the pH of the formulations were measured at different time points as well as being analyzed by HPLC for atenolol content to report any major drug degradation. This third group of atenolol syringes prepared with the optimized formula were prepared having two different pH's (pH 7.37 and 7.71) and were stored at room temperature for 45 days.

Sample collection and analysis for all stability studies were performed in a similar fashion. Atenolol content in the syringes were measured by HPLC to determine the effect light and pH had on atenolol stability.

RESULTS

The gel in initial syringes that were prepared were tested for atenolol concentrations gels and were within acceptable limits for production criteria by the USP compounding guidelines (USP 800). There was no considerable decrease in the atenolol concentration in the topical gel formulations F3, F5, and F6 after one month, 3 months and 6 months of storage (Table 5.1). The loss in the atenolol concentrations was insignificant for up to 6 months. Formulation F3 stored at 37oC had the largest decline from 98.5 percent to a final 97.74 percent of atenolol present at the beginning of the storage trial and represent 2.26% loss of atenolol concentration. This decline was within the USP 800 guidelines for acceptability. The other test formulations saw less than 0.5 percent decline in atenolol content over the six months whether stored at room temperature or at 37 °C. Formulation 6 declined from 98.9 % to 98.4 % over 6 months of storage in both room temperature and elevated temperature. Based on these results atenolol gel formulations maintains stable atenolol concentrations up to 6 months, which is adequate for the veterinary medicine use.

Condition	Temperature	Initial % atenolol	A month % atenolol	3 months % atenolol	6 months % atenolol
1	Room temperature	F3=98.5 F5=99.1 F6=98.9	F3=98.4 F5=98.9 F6=98.7	F3:98.32 F5=98.8 F6=98.4	F3=98.12 F5=98.73 F6=98.35
2	At 37.5 °C	F3=98.5 F5=99.1 F6=98.9	F3=98.1 F5=99.5 F6=98.7	F3=97.8 F5=98.9 F6=98.5	F3=97.74 F5=98.63 F6=98.49

Table (5.1) Stability study of atenolol test formulations under different temperature conditions.

A second stability test was conducted using optimal transdermal atenolol gel formulation at different pH levels (6.5, 7, 7.4, 7.7) keeping all other constituents and amounts the same. The stability study was designed to expose half of the atenolol gel syringes to light while the other half were protected from light exposure. It was found that higher pH levels accelerated atenolol degradation independent of light exposure (Figures 5.1 and 5.2 and Tables 5.2 5.3).

LIGHT GROUP	DAY (0) Atenolol conc (mg/ml	DAY (7) Atenolol conc(mg/ml)	DAY(14) Atenolol conc(mg/ml)	DAY(21) Atenolol conc(mg/mg)	DAY (28) Atenolol conc(mg/ml)
PH 6.5	124.9	123.3	123.1	123	123
PH7	125.1	124.6	124.5	124.2	123.97
PH7.4	125.2	124.9	124.8	124.3	124.22
PH 7.7	124.3	120.3	119.7	105.6	99.24
DARK GROUP					
PH6.5	124.7	123.02	123.3	123.2	123.106
PH7	124.9	123.25	123.05	123	122.96
PH7.4	125	124.9	124.3	124	123.88
PH 7.7	124.8	120.6	119.4	103.3	97.70

Table 5.2 Stability revealing the effect of pH and light exposure on atenolol stability.

Syringes stored at pH 6.5, 7.0 and 7.4 exhibited small declines in atenolol concentrations over 28, 60 and 90 days. Atenolol syringes stored at pH 7.7 whether stored in light or dark saw a decrease in drug concentration of 15-19% at 21 days (light and dark respectively) and continued decreasing to a final 31-32% (light, dark respectively) loss for the entire 90 days.

Light group	Atenolol concentration at 60 days (mg/ml)
pH 6.5	122.79
pH 7	123.53
pH 7.4	123.82
pH 7.7	85.41
Dark group	
pH 6.5	122.74
pH 7	121.80
pH 7.4	122.95
pH 7.7	86.33

Table 5.3 Presents the results of atenolol concentration in syringes stored for 60 days. Atenolol formulation had different pH's and light storage conditions.

Light group	pH after 90 days	Atenolol conc. (mg/ml) 90 days
pH6.5	6.6	121.57
pH 7	7.06	123.15
pH 7.4	7.45	123.01
pH 7.7	7.71	78.45
Dark group		
pH6.5	6.6	122.35
pH7	7.04	121.4
pH7.4	7.37	122.08
pH7.7	7.67	81.21

Table 5.3 continued. Presents the results of atenolol concentration in syringes with different pH and light storage conditions after 90 days of storage.

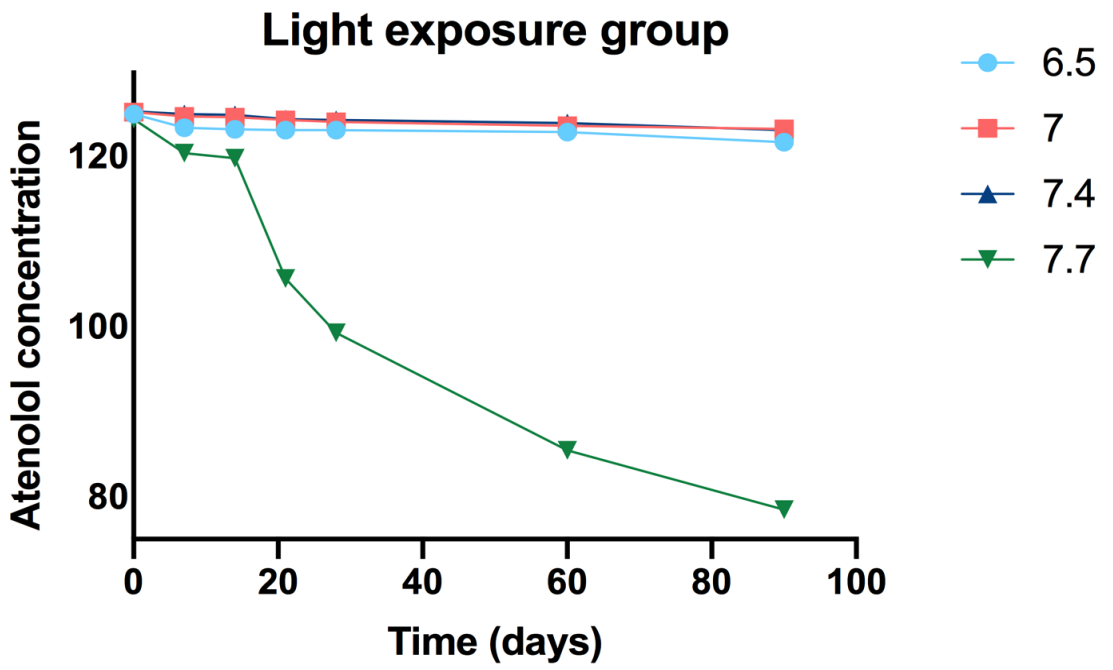

Figure (5.1) The effect of pH and light exposure on atenolol stability over time

Figures 5.1 and 5.3 visually depicts this loss of atenolol concentration in the syringes.

Atenolol stability is affected by pH and the best range that keeps atenolol concentration is less than 7.4. The light has no effect on atenolol stability.

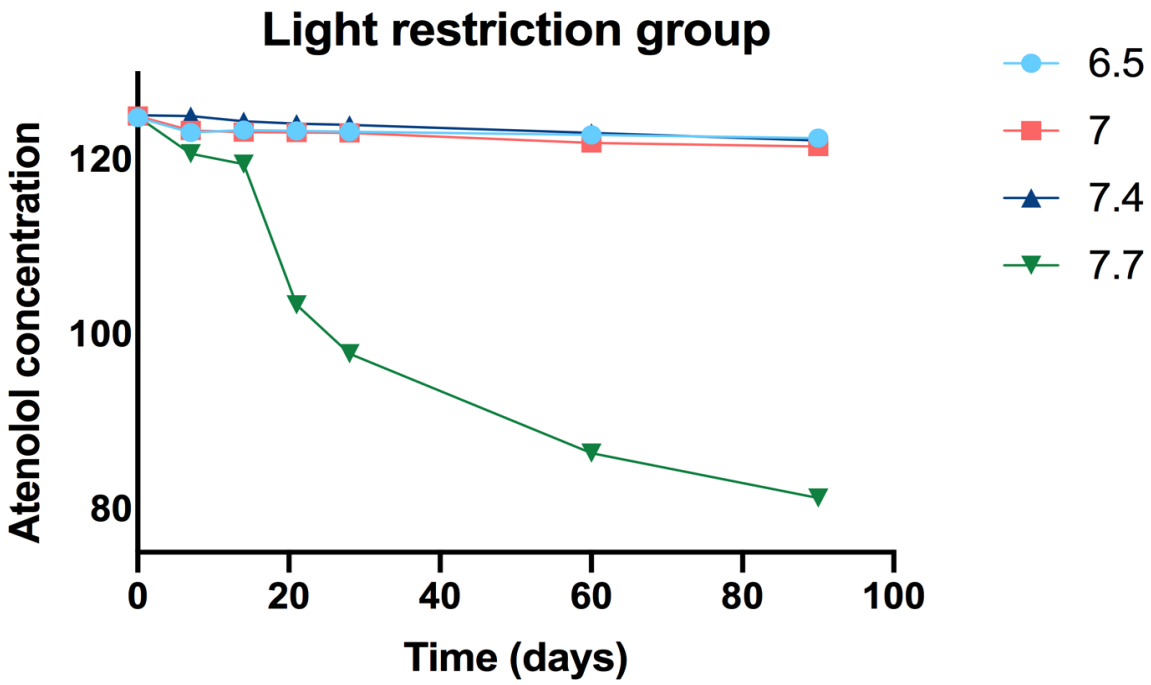

Figure 5.2 The effect of pH on atenolol concentration in syringes that were stored in the dark.

Figure 5.2 shows the effect of pH on the atenolol alone. This figure as the previous Figure 5.1 illustrates atenolol stability is dependent on the pH of the vehicle. Stability results suggest that the pH is an important component that controls the atenolol availability in the formulation. According to this stability study the pH of the formulation should be measured before drug dispensing and application.

Formulation code	Vet. med formulation pH / atenolol conc (mg/ml)	Pharmacy college formulation pH and atenolol assay
0 days	pH =7.71 / (125.03 mg/ml)	pH =7.37, (124.903 mg/ml)
15 days	pH =7.87 / (124.93 mg/ml)	pH =7.23, (124.84 mg/ml)
30 days	pH=7.98 / (123.85 mg/ml)	pH =7.10, (124.809 mg/ml)
45 days	pH= 8.01/ (120.03 mg/ml)	pH =7.09, (124.790 mg/ml)

Table 5.4 Atenolol Stability Study Comparing a Compounding Pharmacy veterinary medicine formulation of atenolol to Optimized Formulation (6) used in the study to treat cats.

Table 5.4 presents the comparison study between formulation versus the formulation base that the College of Veterinary Medicine College usually uses for transdermal drug administration. The results in this table indicate higher concentrations of atenolol in the optimized formula are stable for 45 days compared to the formulation that is often used in veterinary medicine. The atenolol loss in the optimized formula of the current study after 45 days was 0.113 mg (0.09 % loss) of the atenolol initial concentration while the loss for other formulation (veterinary formulation) was 5 mg (4%) compared to the starting concentration.

DISCUSSION

Formulation 6 was tested for stability of atenolol in the syringes and compatibility. This stability test was done after choosing the Formulation 6 as the optimized formula to be used for the clinical trial phase of this study. The results showed no significant degradation in atenolol concentration over a 90 days period.

During the in vivo study, discoloration of the transdermal gel was observed with the second batch of atenolol gel within a month of gel generation. Cats that were administered this batch of the optimal formulation registered sub-therapeutic serum concentrations of atenolol. Considering the administration of the appropriate dose and strong owner compliance that occurred a literature search revealed that changes in pH and light exposure accelerates the degradation of atenolol under experimental conditions (Lina Y et al., 2017). The atenolol concentration and the pH of the gel was tested for this batch after turning yellow, the pH was high and drug concentration was 50% below the initial concentration showing severe atenolol degradation.

A second stability test was conducted using optimal transdermal atenolol gel formulation at different pH levels (6.5, 7, 7.4, 7.7) keeping all other constituents and amounts the same. The stability study was designed to expose half of the atenolol gel syringes to light while the other half were protected from light exposure. It was found that higher pH levels accelerated atenolol degradation independent of light exposure

An important finding is the effect pH has on atenolol stability after formulating a topical dosage form. The measuring pH before dispensing and patient use is critically important to assure the stability of atenolol in the syringe. The results of the second stability analysis

performed revealed the reason for the color change of the formulation. Degradation products of atenolol turns the product brown upon storage. The importance of pH adjustment to the topical product to below 7.4 for atenolol stability is independent of light exposure. Oxidation-reduction reactions are usually pH dependent. Atenolol undergoes this type of reaction in the degradation process, which require a proton (H^+)/ hydroxyl (OH^-) for the reaction to take place (ref). Hence any change in these ions concentration (raising or lowering the pH) will alter the rate of the reaction. The data from our study suggests a pH of 7-7.4 is adequate to minimize atenolol degradation over a 3 months period. It is a critical to measure the formulation's pH to verify the product made will be prior to dispensing.

This study determined the optimal hydrogen ion concentration (pH) that keeps the atenolol stable in the transdermal formulation. The pH of gel is critically important to the stability of atenolol in the syringe. The second stability study addresses the reason for the formulation deterioration. The results indicate that pH of 7 can minimize atenolol loss in the gel. The importance of pH adjustment to the topical product to below 7.4 on atenolol stability is independent of light exposure. These stability results suggest that the pH is an important component that controls atenolol availability in topical administration of this formulation. From this stability study the pH of the formulation should be monitored before the drug dispensing and patient application.

CONCLUSION

Atenolol stability in the designed formulation was studied up to 6 months. There was no significant change in the atenolol content in the formulation. The stability studies indicate minor loss in the atenolol concentration in the designed formulation both in room temperature and at 37.5 °C.

This finding supports that the atenolol stability is attainable up to 6 months, a time period is convenient for veterinary use. All initial atenolol gel concentrations in the gels tested were within acceptable limits for production criteria by the USP compounding guidelines (USP 800). There was no considerable decrease in the atenolol concentration in the topical gel formulations F3, F5, and F6 after one month, 3 months and 6 months of storage.

This decline was within the USP 800 guidelines for acceptability. The other test formulations saw less than 0.5 percent decline in atenolol content over the six months whether stored at room temperature or at 37 °C. Based on these results atenolol formulation maintains the atenolol stability up to 6 months which is adequate for the veterinary medicine use.

The second stability study addresses the reason for the formulation deterioration. The pH is a critical component of the formulation's stability, and pH (target pH of 7) should be measured by pharmacists prior to dispensing this compounded medication.

This study determined the optimal hydrogen ion concentration (pH) that keeps the atenolol stability in the transdermal formulation. The formulation's pH is critically important to the stability of atenolol in the dispensed syringes. The results indicate that pH of 7 produces

the minimum level of atenolol loss. The importance of pH adjustment to the topical product to below 7.4 on atenolol stability is independent of light exposure. Exposure of the atenolol gel to light may have deleterious effects but will occur after 6 months of storage. Finally, this new optimized formulation can keep atenolol concentration stable for reasonable shelf life when compounded for patient use.

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CHAPTER 6

CONCLUSIONS

CONCLUSIONS

The ingredients in the optimized formulation produced the enhancement that was desired to produce a sufficient amount of atenolol that can diffuse through cat skin. The enhancers that were used in the optimized formulation were powerful and improved the atenolol permeation more than the increase in atenolol solubility in the formulation. The enhancers used were 5% Polysorbate 80 (a surfactant), Propylene glycol, Glycerol added at 15 % each as both enhancers are also solvents and can provide occlusive effects that helps atenolol penetration by increasing the skin hydration. Ethanol was added as a solvent enhancer at 10% concentration. Dimethyl-isosorbide (DMI) was added at 15 % concentration that provided more skin mobilization and enable more atenolol penetration. The formulation was adjusted in terms of its texture by limiting the amount of the Carbomer (the gel base) to 0.75% so the required amount of softness is obtained. The 6 formulations that were designed for the current study were tested and evaluated on the different membranes (synthetic, cloned human epidermis and cat skin) to determine the effective formulation. Atenolol concentrations were assayed for estimating the total amount of the diffused atenolol through cat skin. It was found that the concentration of atenolol 2 hours after application of formulation 6 to cat skin was 35.5 µg/ml. This correlates to 491.12 µg penetrating over 2 hours. The therapeutic concentration of atenolol is 260 ng/ml (Quinone et al., 1996) and by considering, atenolol has a volume of distribution of ~1000 ml in cat, therapeutic concentrations of atenolol can be attained using the optimized formula.

The feasibility of producing a topical atenolol gel formulation showed that an atenolol transdermal delivery system can be used in veterinary medicine for the treatment of feline heart disease.

The results of the percent cumulative drug release were examined in accordance to the kinetic models such as Zero-order, First-order, Higuchi equation, Korsmeyer–Pappas equation and Hixson–Crowell equation. In Zero order, R^2 was calculated for each formulation according to the different kinetic models the values of regression coefficient was R^2= 0.9012 for formulation 3, Formulation 5 R^2= 0.9137 and Formulation 6 R^2= 0.92. In First order, R^2= 0.855 for Formulation 3 and R^2= 0.791 for formulation 5 and R^2= 0.816 for formulation 6. The graph of the data using Higuchi model was plotted. The cumulative percentage drug released versus square root of time for each formulation through cat skin was not linear and the value of regression coefficients of R^2= 0.607 for formulation 3, and R^2= 0.739 for formulation 5 and formulation 6 value of regression coefficient was R^2= 0.701 were considerably less than the R^2 values for the zero-order model. In Hixon-Crowell model, the value of regression coefficient was R^2= 0.541 for formulation 3 and R^2= 0.593 and R^2= 0.653 for formulation 6. The zero-order release model provided the best explanation of drug diffusion through the membranes. The Korsmeyer-Peppas n value for the first phase was 0.8571 for the initial burst flux indicating a fickian diffusion process of a drug solution. The n value for the second flux phase was 0.2616. This indicates that dissolution of the drug from the drug particle occurs before diffusion through the cat skin. Erosion of the drug particle may also be involved in this process.

Three different atenolol formulations were developed in order to compare their performance as potential vehicles for achieving effective skin delivery of atenolol. The best formulations selected were tested to evaluate their ability to deliver the drug into the human skin in comparison to a simple aqueous atenolol solution containing the same amount of drug (1% w/v). All the atenolol formulations markedly ($p < 0.001$) improved the amount of drug that penetrated through the skin layers compared to the simple aqueous solution. A minimum of 700% increase atenolol penetration through cat skin in the case of Formulation 3, to 750% for Formulation 5 and up to a maximum of 900%, in the case of Formulation 6 was observed.

In particular, Formulation 6 containing DMI 15% as drug carrier showed greater diffusion of atenolol though cloned human skin and cat skin and provided better consistency terms of drug delivery. It also gave rise to the highest increase in drug penetration ability through skin, probably due to the simultaneous presence in the composition of ethanol and polysorbate 80, propylene glycol, glycerol and 15 % DMI all acting as permeation enhancers**.**

The results of the similarity factor tests of the three selected formulations through cat skin were inconclusive as at best. Formulation 6 produced the highest flux of atenolol through cat skin and was used as the reference formulation. (f_2) indicated that no differences were detected between all the formulations. The evaluation studies that were carried out show the discrimination criteria needed to differentiate between the different formulations' diffusion through the different barriers (synthetic, cloned epidermis and cat skin) in this study was not helpful to indicate which formulation is the optimal one. However, Formulation 6 did have consistently higher pAUC and AUMC values. This occurred with

cat ear skin and cloned human skin diffusion data. In such cases the choice of the best formulation is often left to the formulator. The formulation was determined by selecting which formulation produced the greatest atenolol diffusion through cat skin. Formulation 6 not only had the greatest diffusion through cat skin it also provided more consistent release as observed in the atenolol permeation profiles.

The first group of cats in this clinical trial were administered a dose of 12.5 mg per cat q12h and failed to provide therapeutic serum atenolol concentrations. However, this was expected as a lower dose of atenolol was intentionally used to start the study with. The use of a transdermal formulation as direct substitution for oral dosages has been previously documented to provide less consistent and lower plasma concentrations (Hoffman SB et al., 2002). In order to get a therapeutic level of a drug by transdermal route the dose should be higher than that used for oral dose. It is also well known that the transdermal formulations provide safe and desired advantages regarding the avoidance of the hepatic first-pass metabolism and easier administration than oral medications in cats.

The major increase in the atenolol dose to 25 mg per day per cat and the dosage regimen (q 12h) that was used in this study was still within the safety margin of the dose of atenolol. The dose used in this study was increased to 25 mg q12h yielding a greater atenolol concentration and was therapeutically sufficient. According to (Macgregor JM et al., 2008) the beta-blocking effects of oral atenolol using isoproterenol challenge starts at the 260 ng/mL, while serum atenolol concentrations of 42 ng/mL did not result in adequate β-blockade. The true therapeutic serum concentration cut-off lies between 42 and 260 ng/ml. The effective therapeutic concentration of serum atenolol in human that produces β-blockade (200-500 ng/mL). Using a 200ng/ml lower limit for the therapeutic concentration

it could be concluded that 4 of 7 cats would have at least one therapeutic at 6hr time point and 6 of 7 at 12 hours post atenolol topical administration achieved therapeutic concentration. At the 3 hours time points 5 of 7 cats would be therapeutic. The results of this study suggest the optimal formulation of atenolol administered transdermally at 25 mg q12h provides therapeutic serum atenolol concentrations and attendant HR reduction in most clinically healthy cats. The constituents of the formulation are commercially available for use by compounding pharmacies.

Atenolol stability in the designed formulation was studied up to 6 months. There was no significant change in the atenolol content in the formulation. The stability studies indicate minor loss in the atenolol concentration in the designed formulation both in room temperature and at 37.5 °C.

This finding supports that the atenolol stability is attainable up to 6 months, a time period that is convenient for veterinary use. All initial atenolol gel concentrations in the gels tested were within acceptable limits for production criteria by the USP compounding guidelines (USP 800). There was no considerable decrease in the atenolol concentration in the topical gel formulations F3, F5, and F6 after one month, 3 months and 6 months of storage.

This decline was within the USP 800 guidelines for acceptability. The other test formulations saw less than 0.5 percent decline in atenolol content over the six months whether stored at room temperature or at 37 °C. Based on these results atenolol formulation maintains atenolol stability for up to 6 months which is adequate for veterinary medicine use.

The second stability study addresses the reason for the formulation deterioration seen in the second batch of atenolol gel produced. The pH is a critical component of the

formulation's stability, and pharmacists should measure pH (target pH of 7) prior to dispensing this compounded medication.

This study determined the optimal hydrogen ion concentration (pH) that keeps the atenolol stability in the transdermal formulation. The formulation's pH is critically important to the stability of atenolol in the dispensed syringes. The results indicate that pH of 7 provides the minimum level of atenolol loss. The importance of pH adjustment to the topical product to below 7.4 on atenolol stability is independent of light exposure. Exposure of the atenolol gel to light may have deleterious effects but will occur after 6 months of storage. Finally, this new optimized formulation can keep atenolol concentration stable for reasonable shelf life when compounded for patient use.

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APPENDIX

Figure A.1-A.6 Show average atenolol diffusion profiles of formulations 1-6 through synthetic membrane respectively.

Figure A.7-A.9 Show average atenolol diffusion profiles for formulation 3, 5 and 6 through cloned human epidermis respectively.

Figure A.10-A.12 Show average atenolol diffusion profiles for formulation 3, 5 and 6 through cat skin respectively.

Figure A.13-A.23 Show Individual atenolol diffusion profiles through cat skin for formulation 3.

Figure A.24-A.33 Show Individual atenolol diffusion profiles through cat skin for formulation 5.

Figure A.34-A.44 Show Individual atenolol diffusion profiles through cat skin for formulation 6.

Figures A..45 through A.53 Show individual atenolol concentration versus time square root o(Higuchi plot) for formulation 6. These graphs as well as all attempts to determine if other

mechanisms for drug release from the applied topical atenolol gel were less than adequate at explaining drug release patterns from the gel.

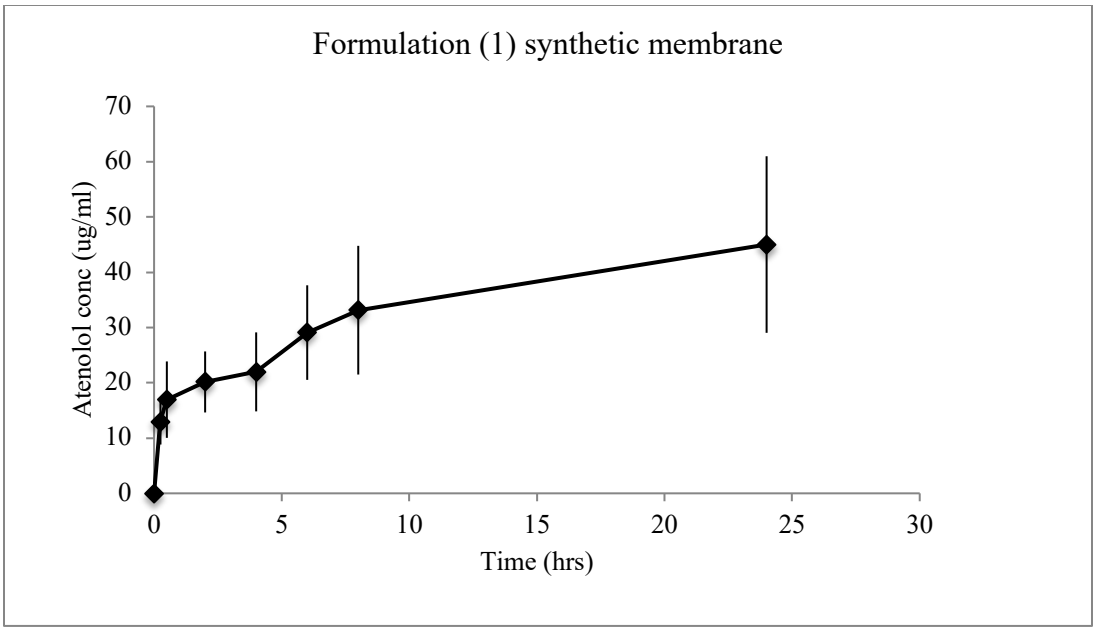

Figure A.1 Atenolol concentration time curve for formulation 1 through synthetic membrane.

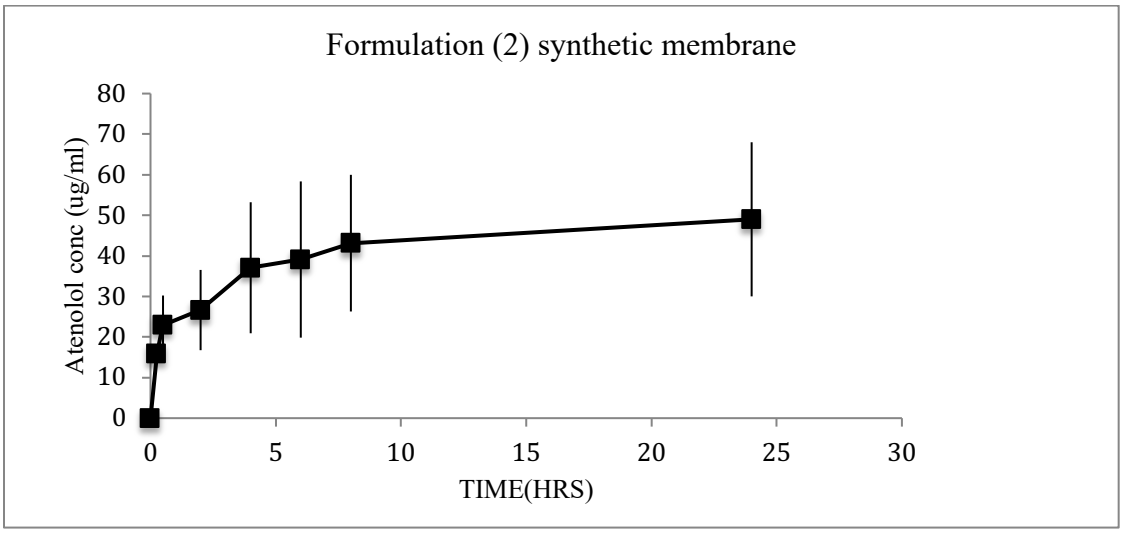

Figure A.2 Atenolol concentration time curve for formulation 2 through synthetic membrane.

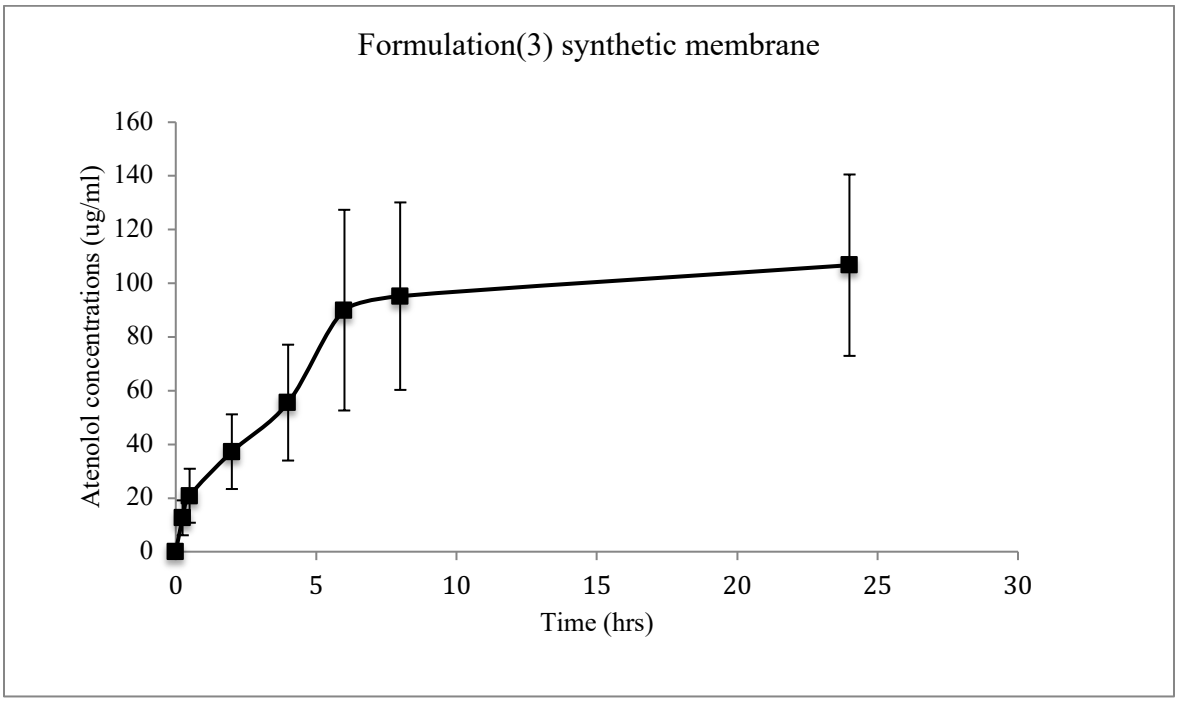

Figure A.3 Atenolol concentration time curve for formulation 3 through synthetic membrane

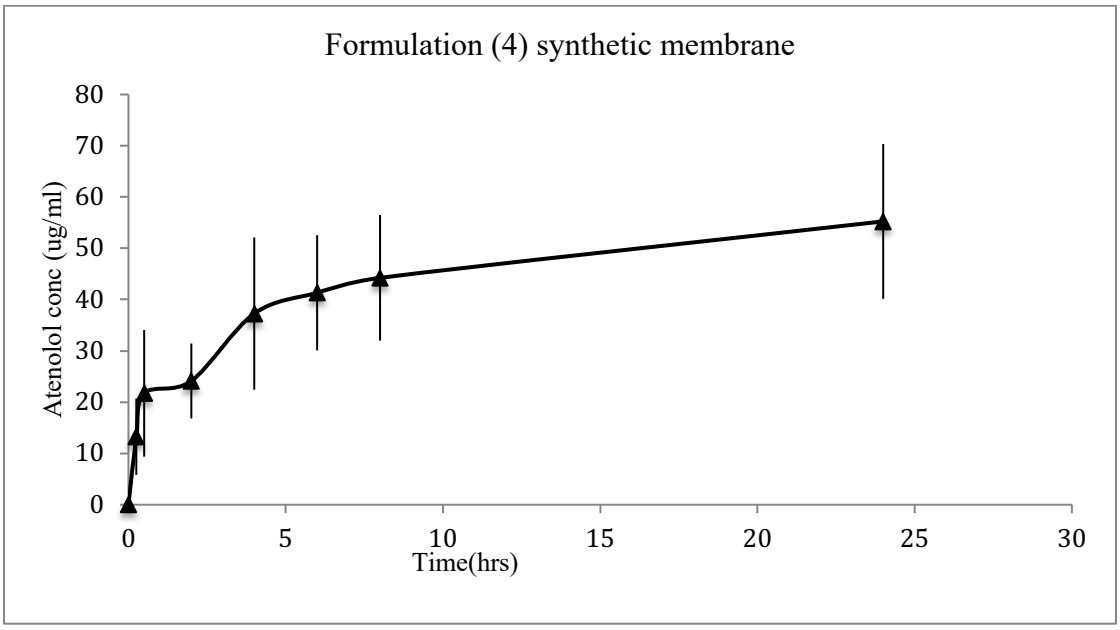

Figure A.4 Atenolol concentration time curve for formulation 4 through synthetic membrane

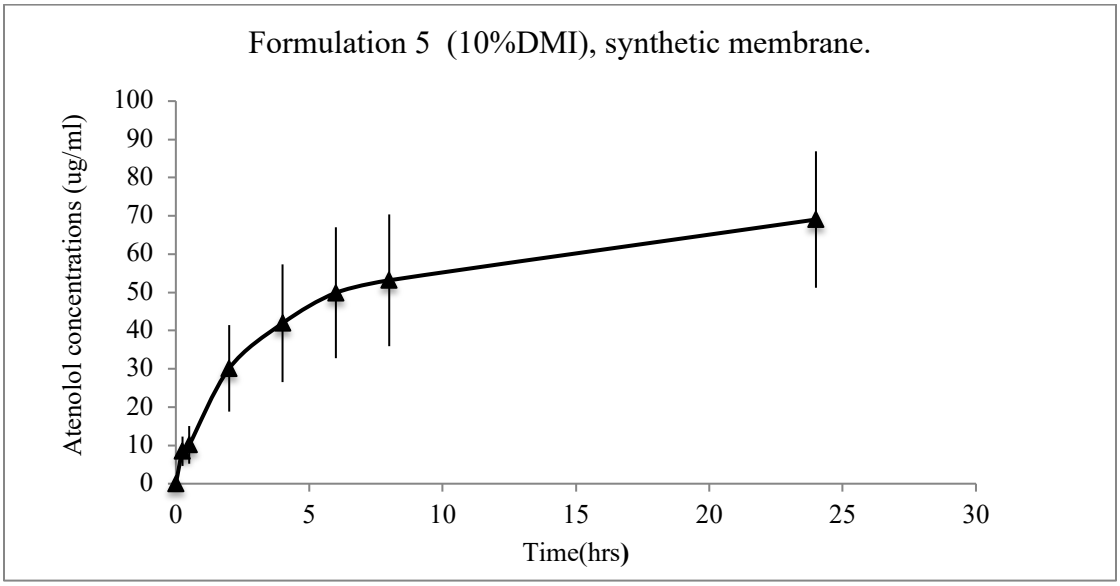

Figure A.5 Atenolol concentration time curve for formulation 5 through synthetic memberane.

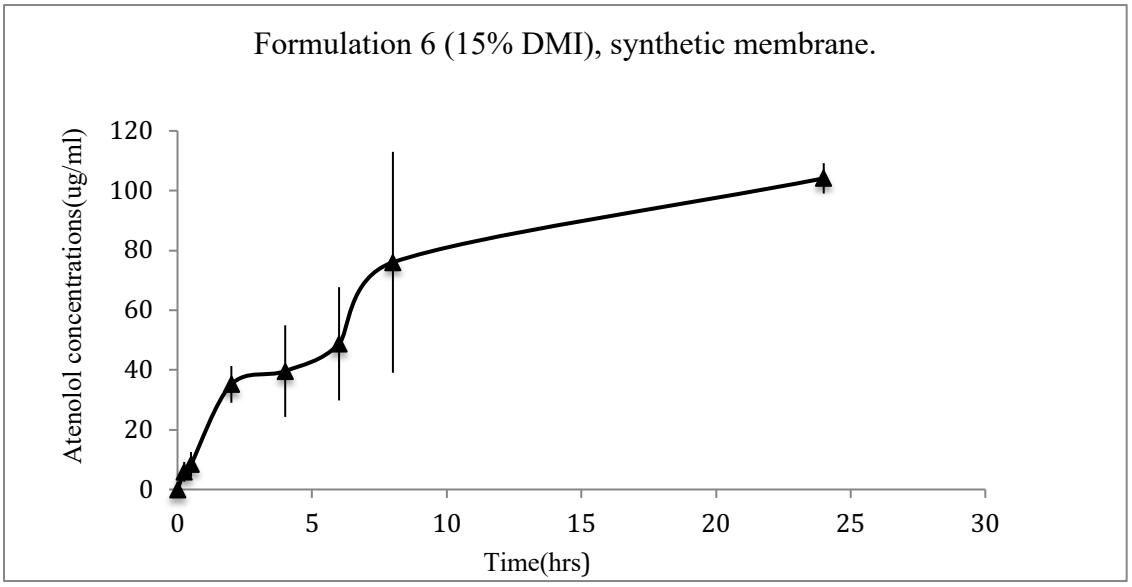

Figure A.6 Atenolol concentration time curve for formulation 6 through synthetic membrane

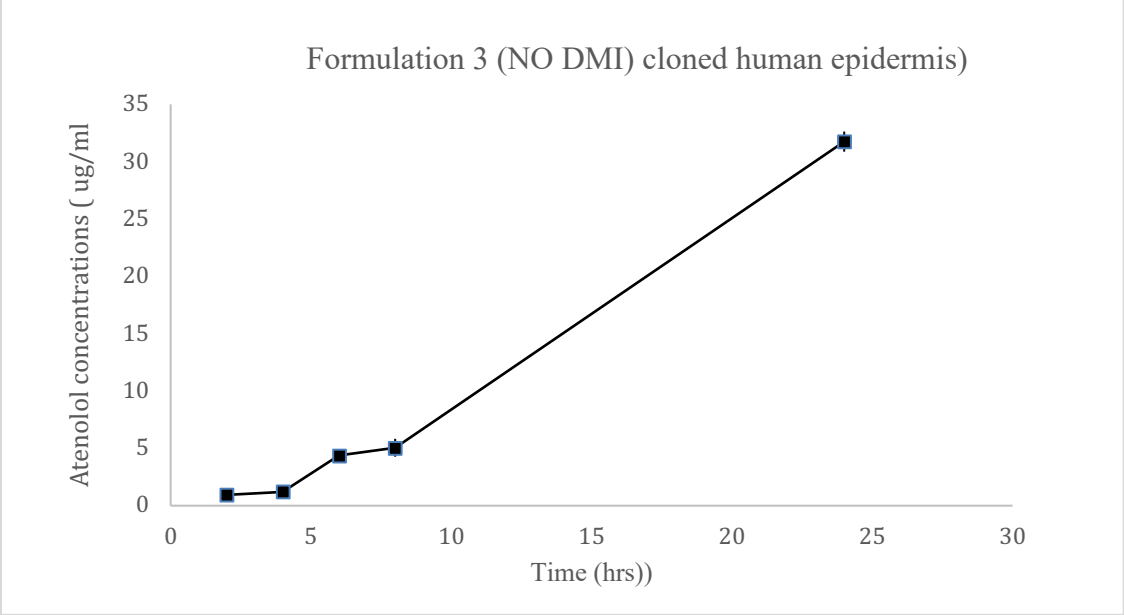

Figure A.7 Atenolol concentration time curve for formulation 3 through cloned human epidermis.

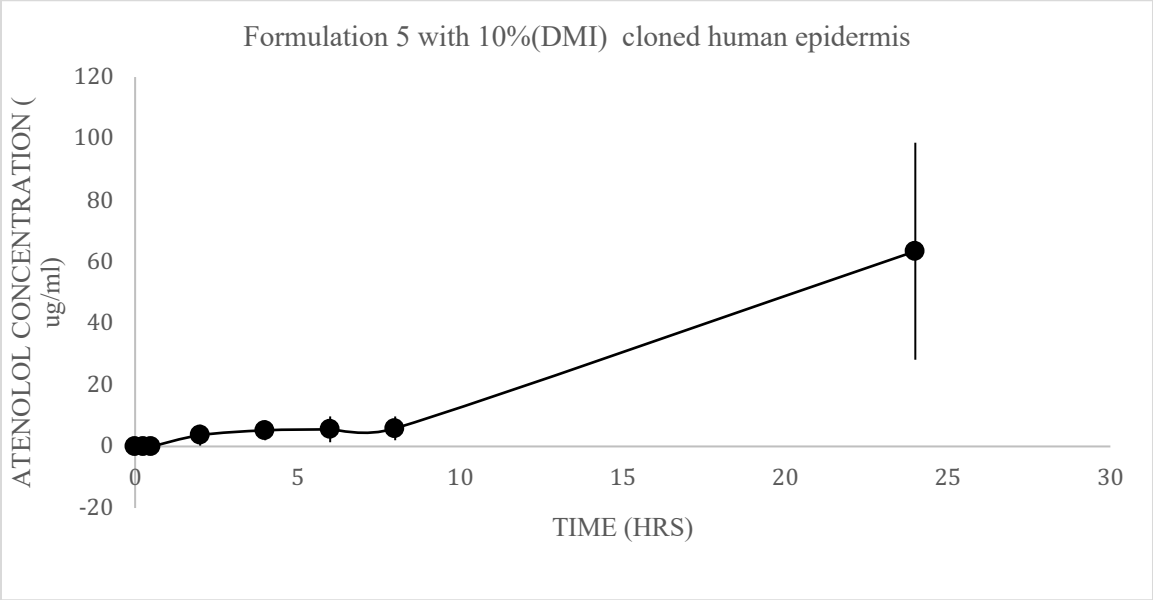

Figure A.8 Atenolol concentration time curve for formulation 5 through cloned human epidermis

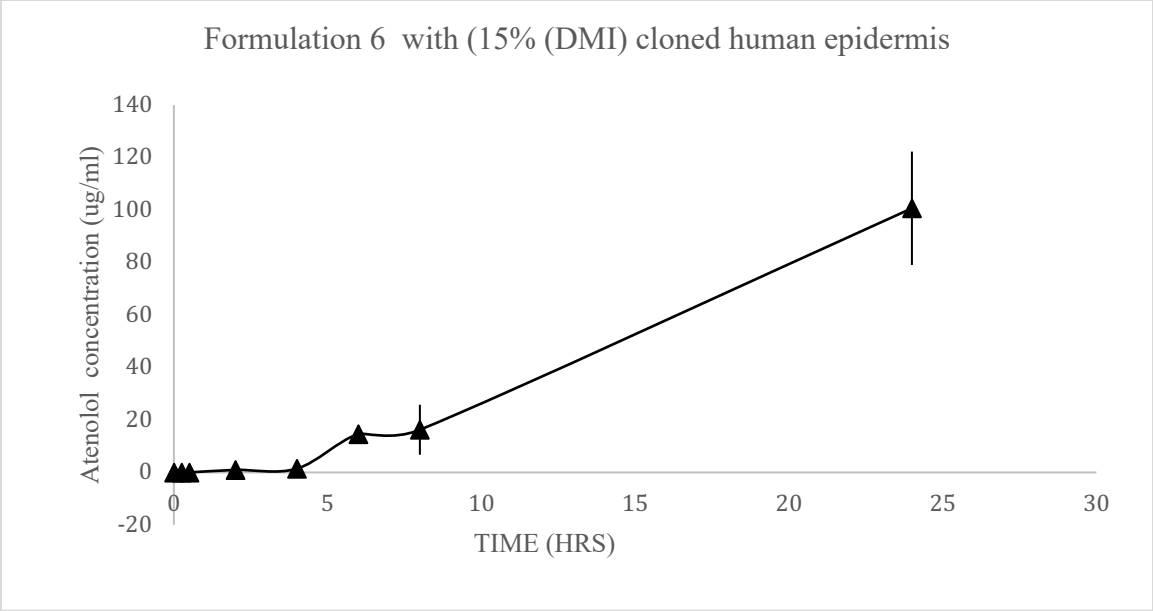

Figure A.9 Atenolol concentration time curve for formulation 6 through cloned human epidermis

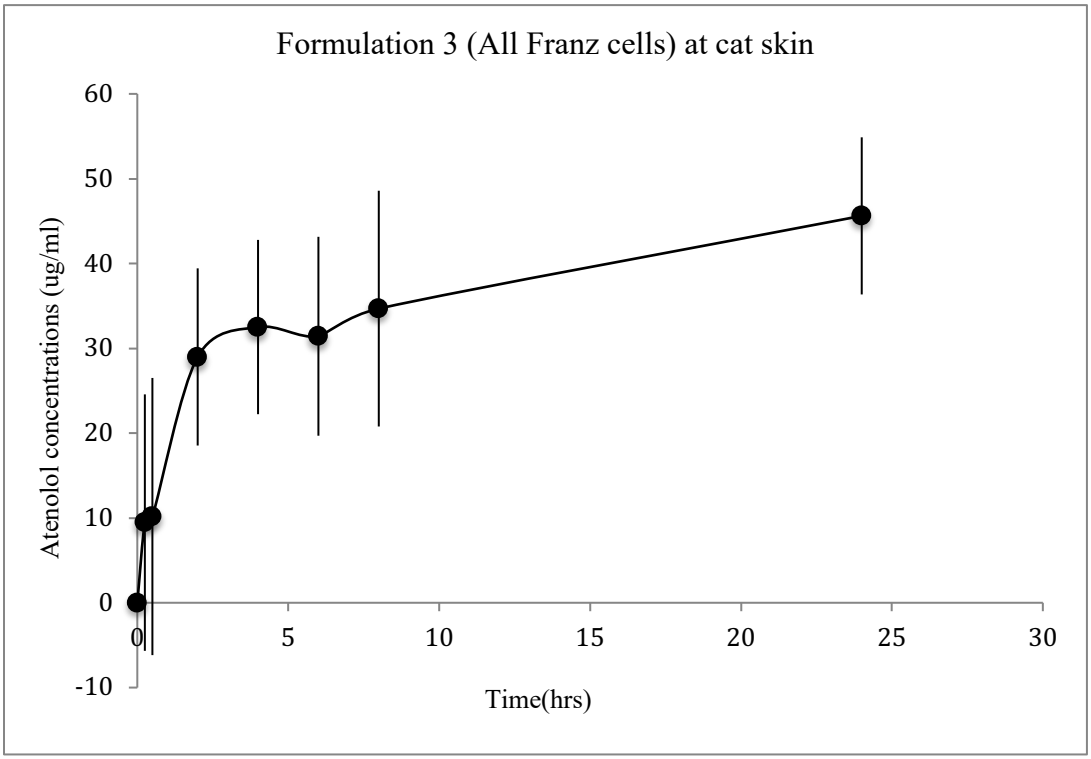

Figure A.10 Atenolol concentration time curve for formulation 3 through cat ear skin.

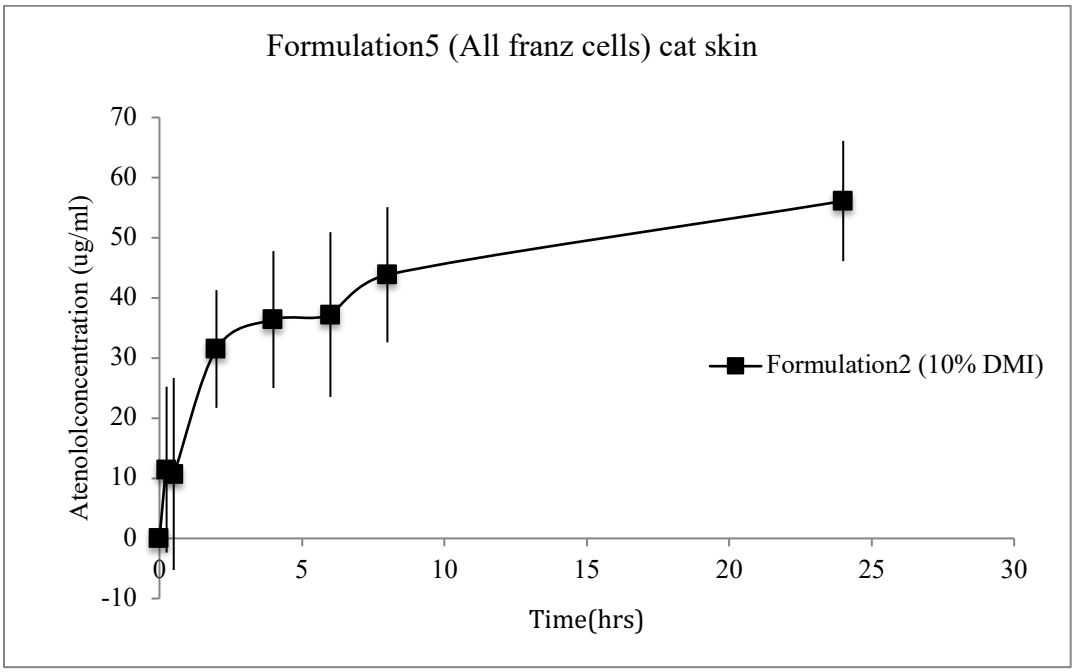

Figure A.11 Atenolol concentration time curve for formulation 5 through cat ear skin.

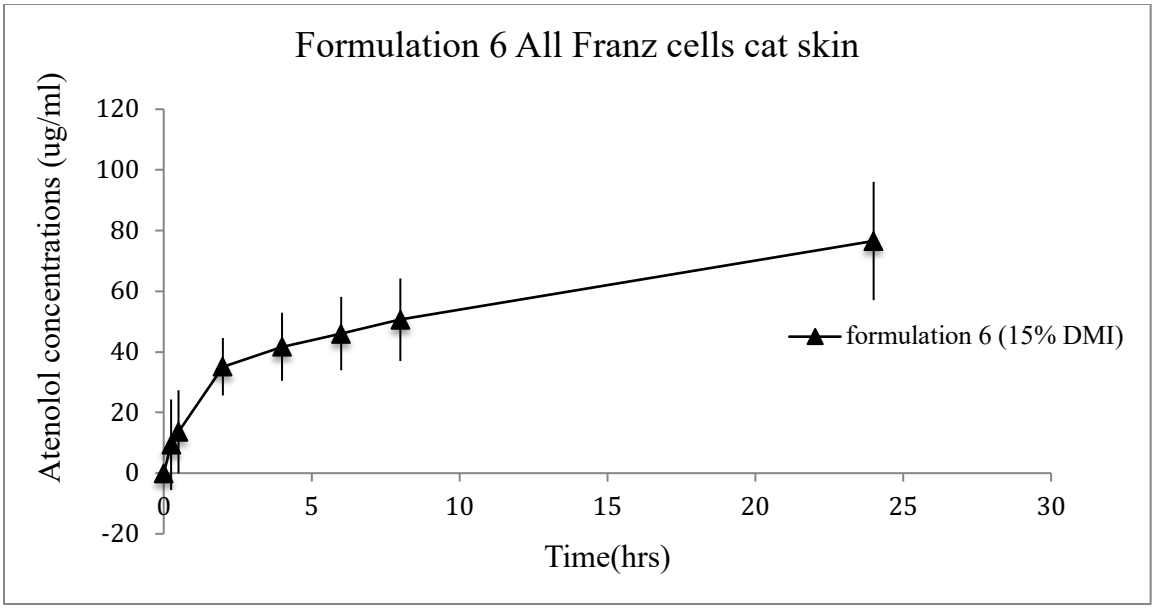

Figure A.12 Atenolol concentration time curve for formulation 6 through at cat ear skin.

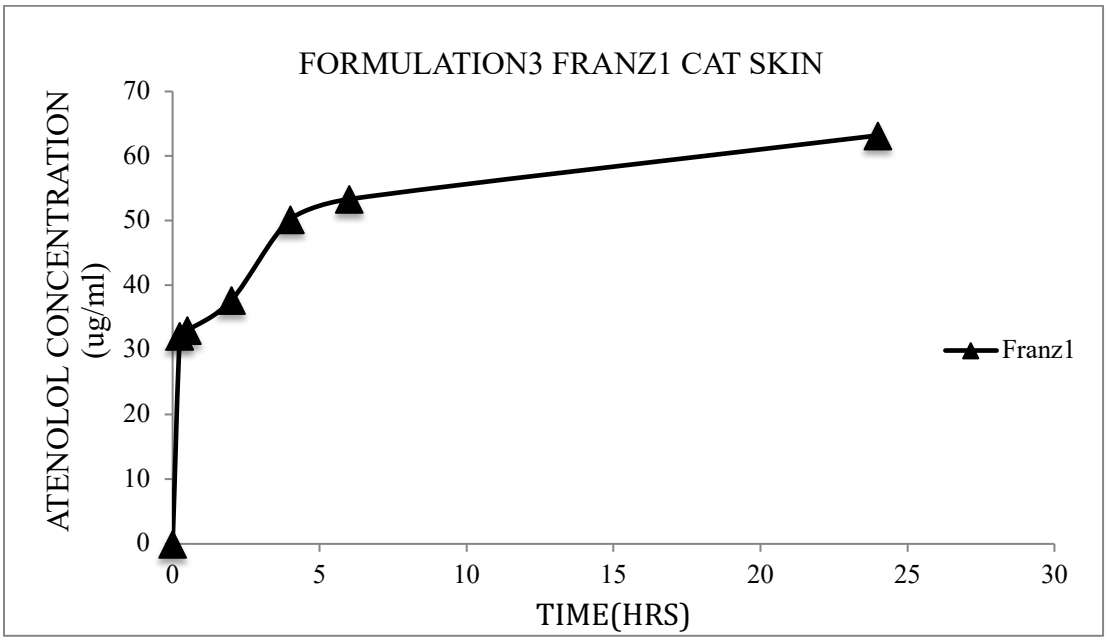

Figure A.13 Individual cat atenolol concentration time curve for formulation 3 through cat skin Franz 1

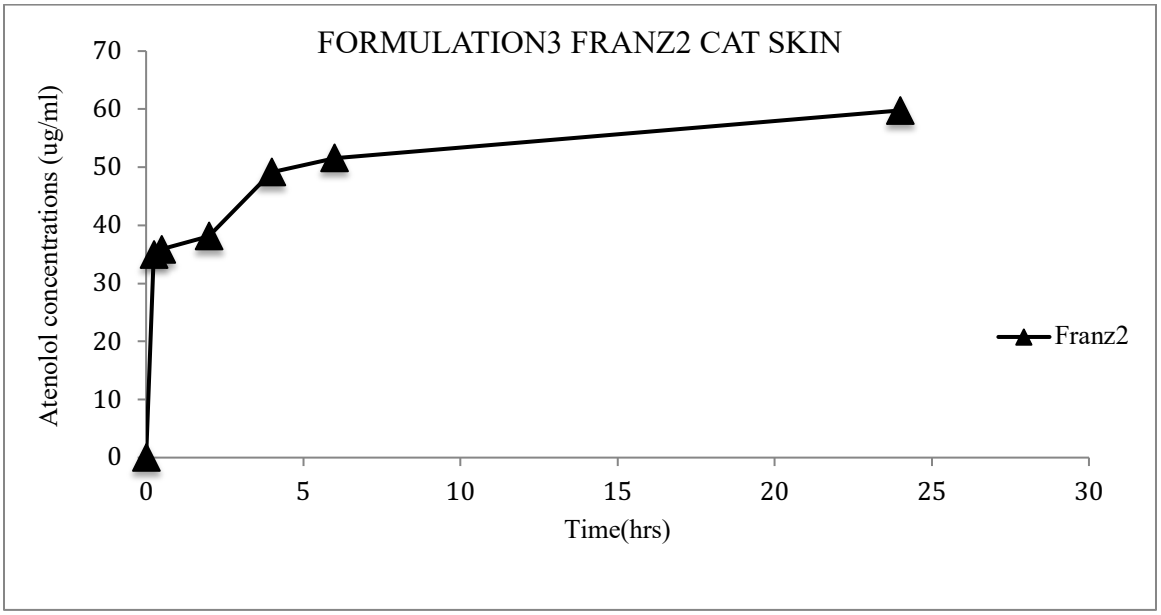

Figure A.14 Individual cat atenolol concentration time curve for formulation 3 through cat skin Franz 2

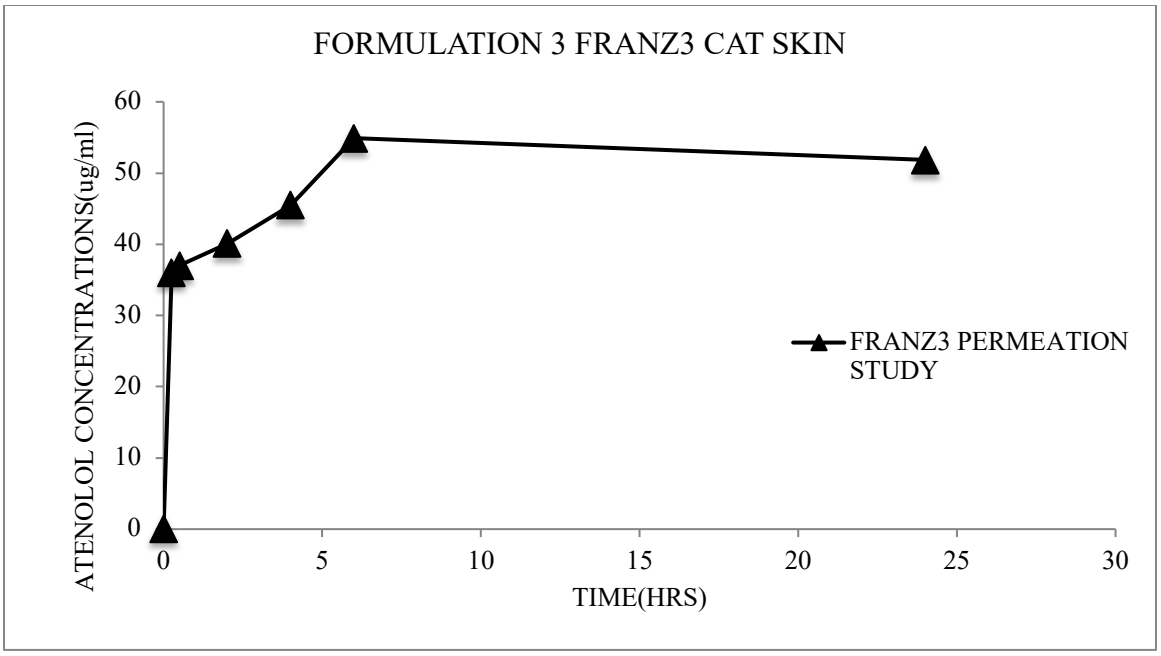

Figure A.15 Individual cat atenolol concentration time curve for formulation 3 through cat skin Franz 3

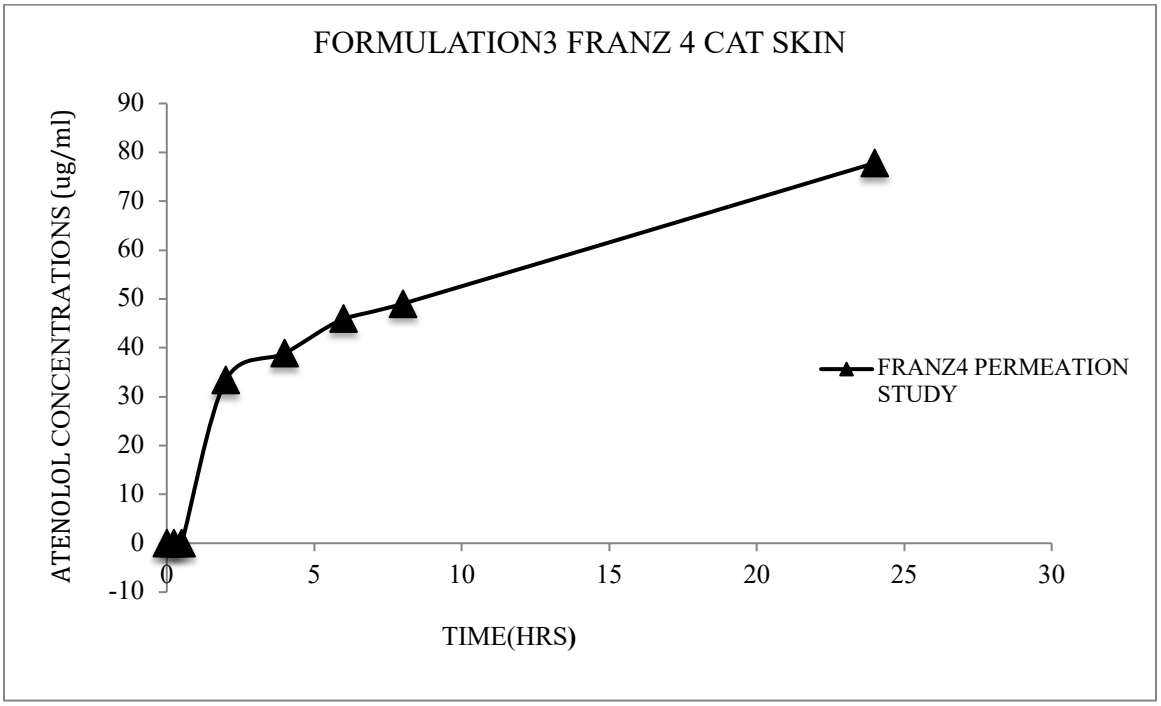

Figure A.16 Individual cat atenolol concentration time curve for formulation 3 through cat skin Franz 4.

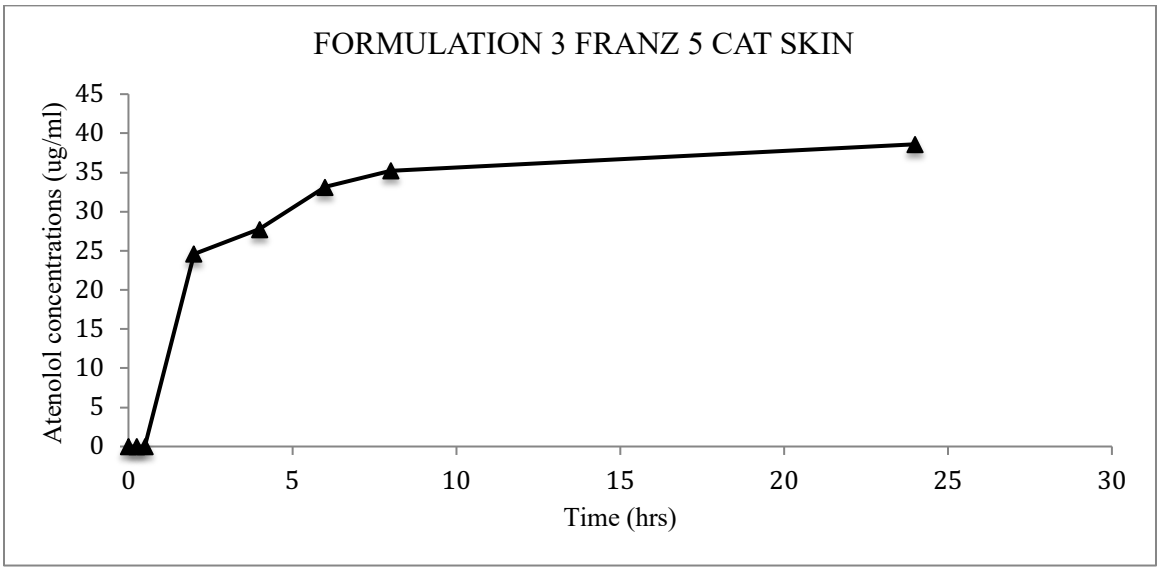

Figure A.17 Individual cat concentration time curve for formulation 3 through cat skin Franz 5.

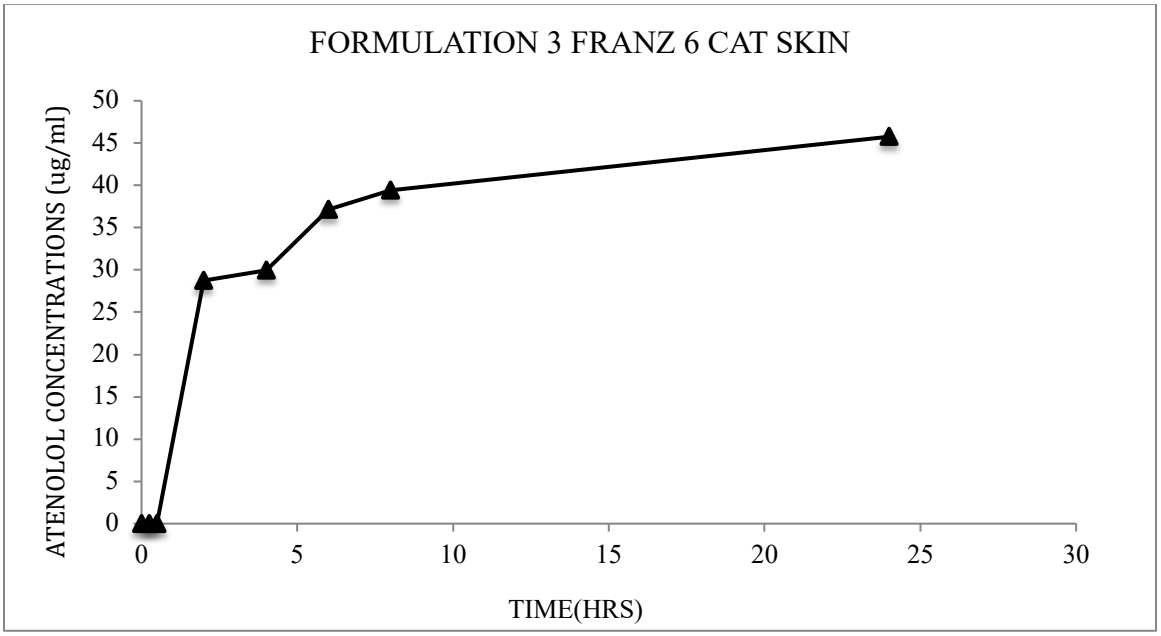

Figure A.18 Individual cat atenolol concentration time curve for formulation 3 through cat skin Franz 6.

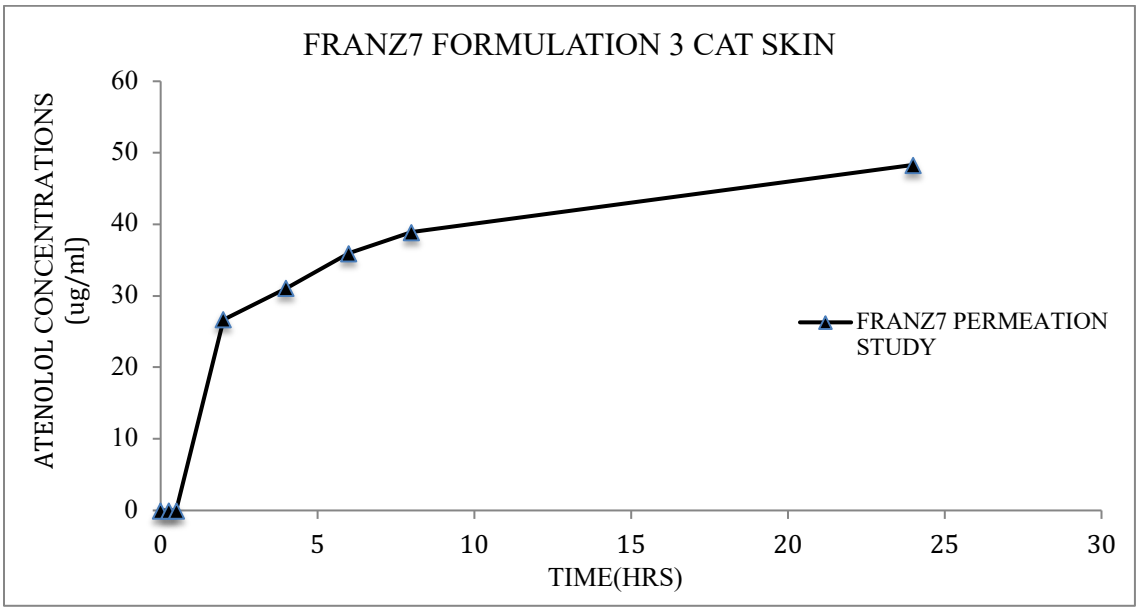

Figure A.19 Individual cat atenolol concentration time curve for formulation 3 through cat skin Franz 7

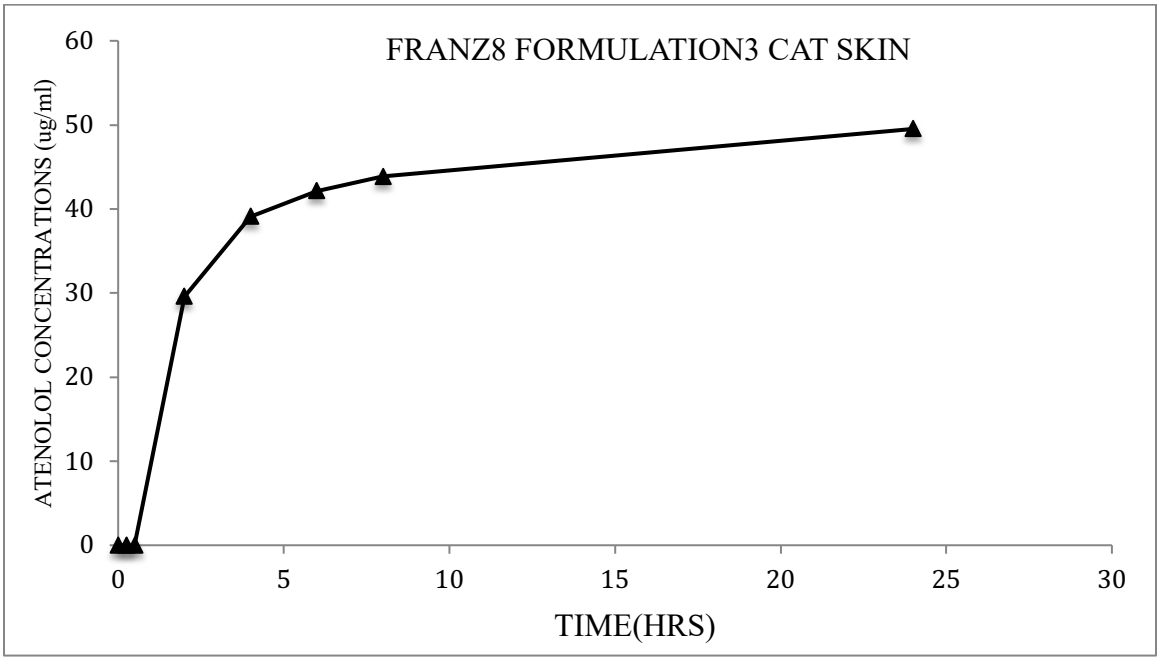

Figure A.20 Individual cat atenolol concentration time curve for formulation 3 through cat skin Franz 8

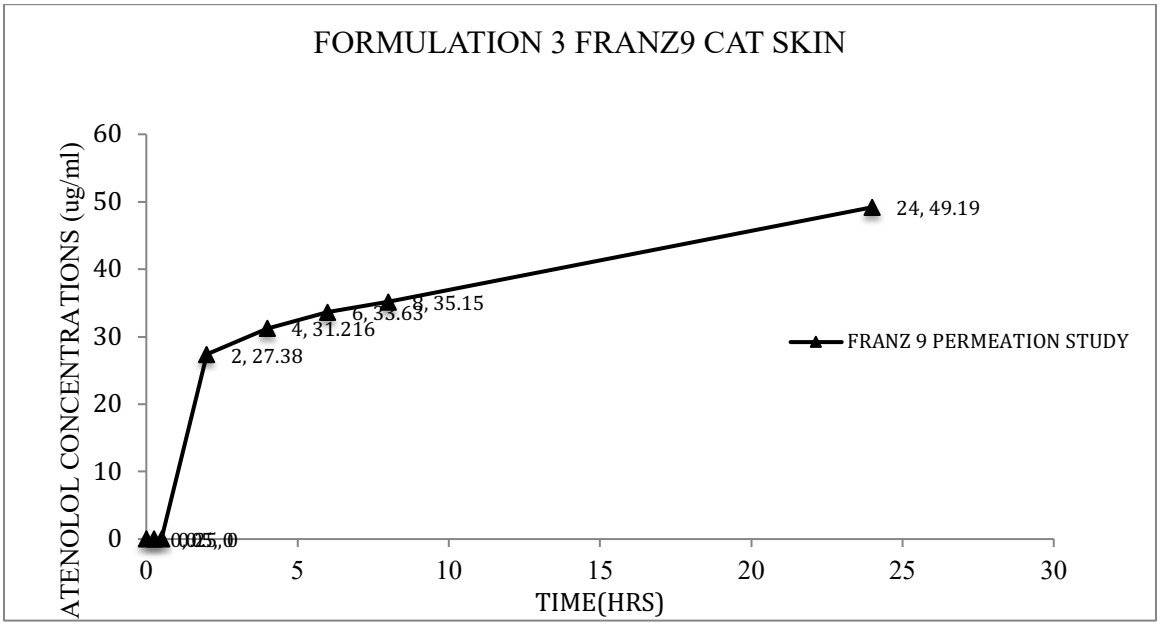

Figure A.21 Individual cat atenolol concentration time curve for formulation 3 through cat skin Franz 9.

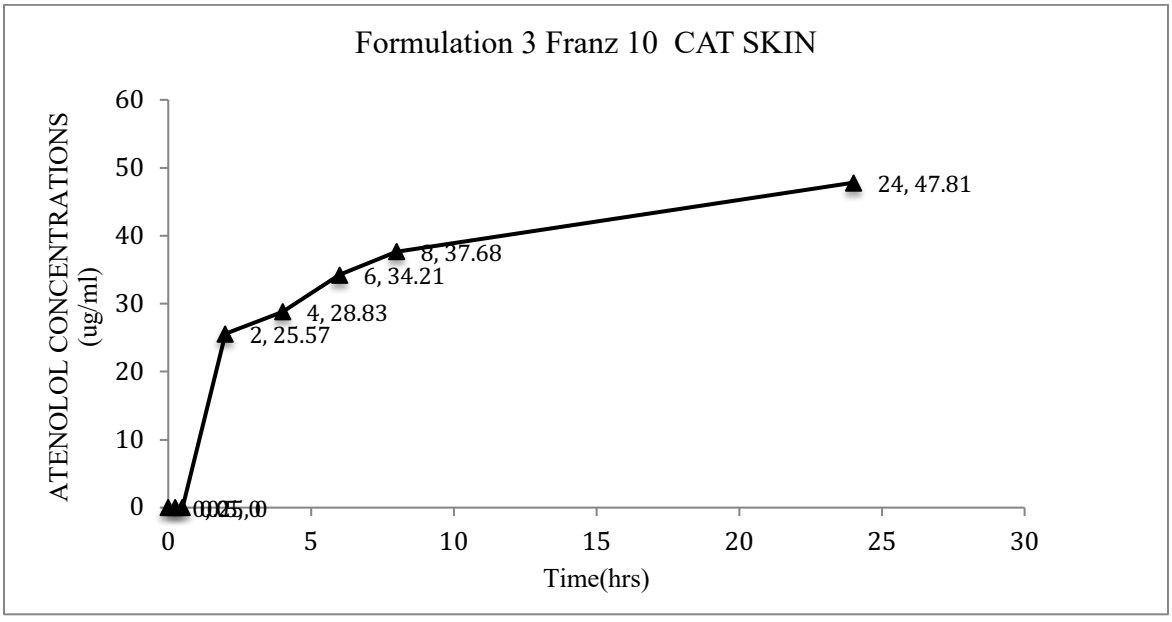

Figure A.22 Individual cat atenolol concentration time curve for formulation 3 through cat skin Franz 10

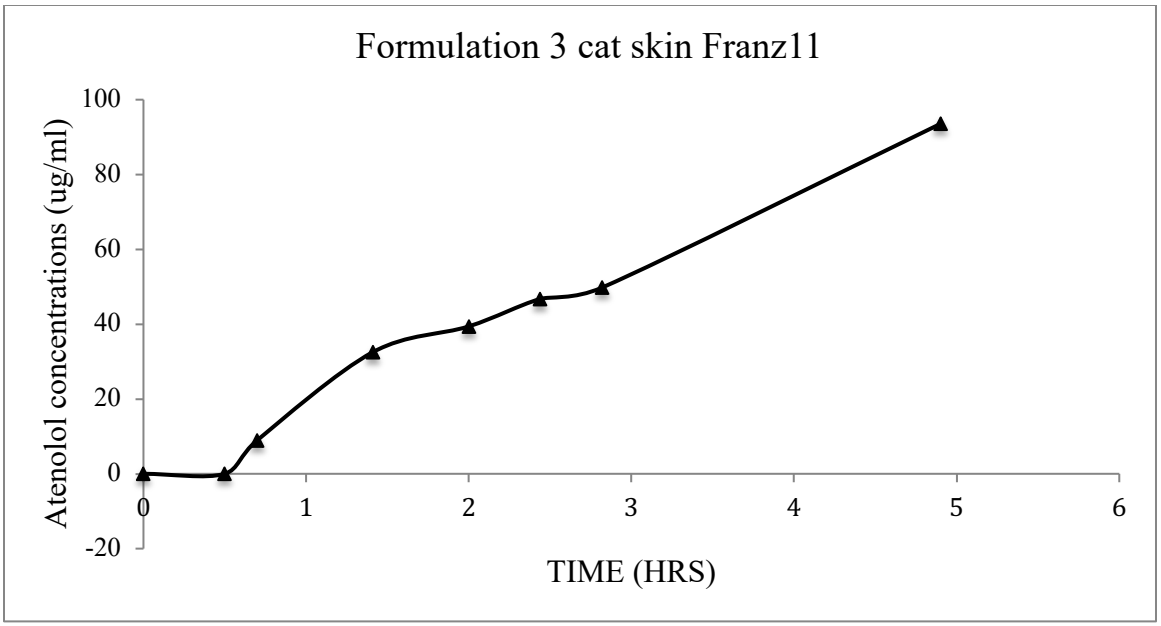

Figure A.23 Individual cat atenolol concentration time curve for formulation 3 through cat skin Franz 11.

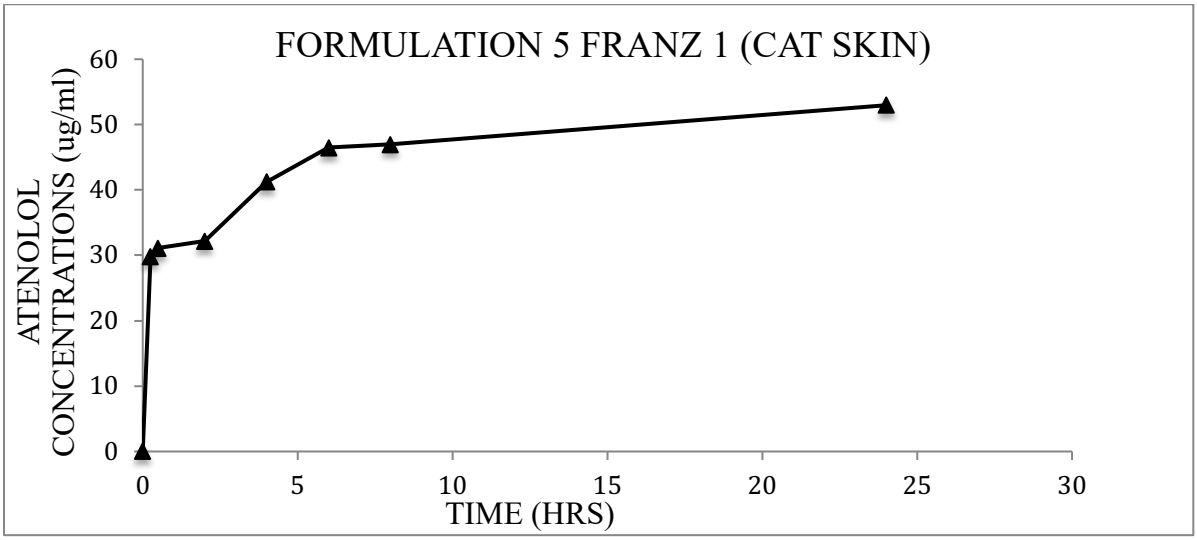

Figure A.24 Individual cat atenolol concentration time curve for formulation 5 through cat skin Franz 1.

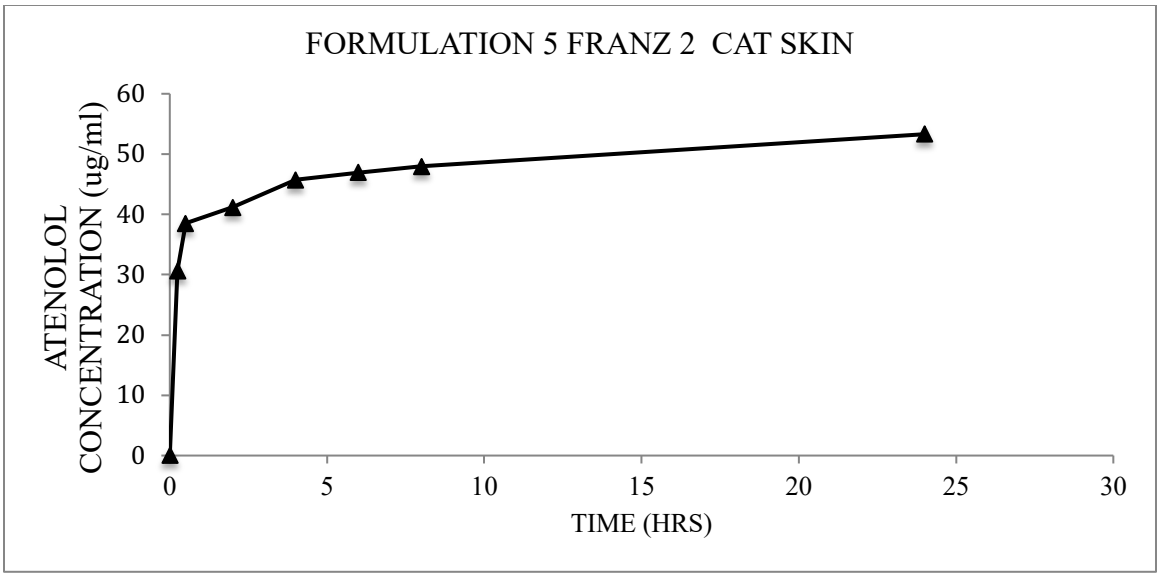

Figure A.25 Individual cat atenolol concentration time curve for formulation 5 through cat skin Franz 2

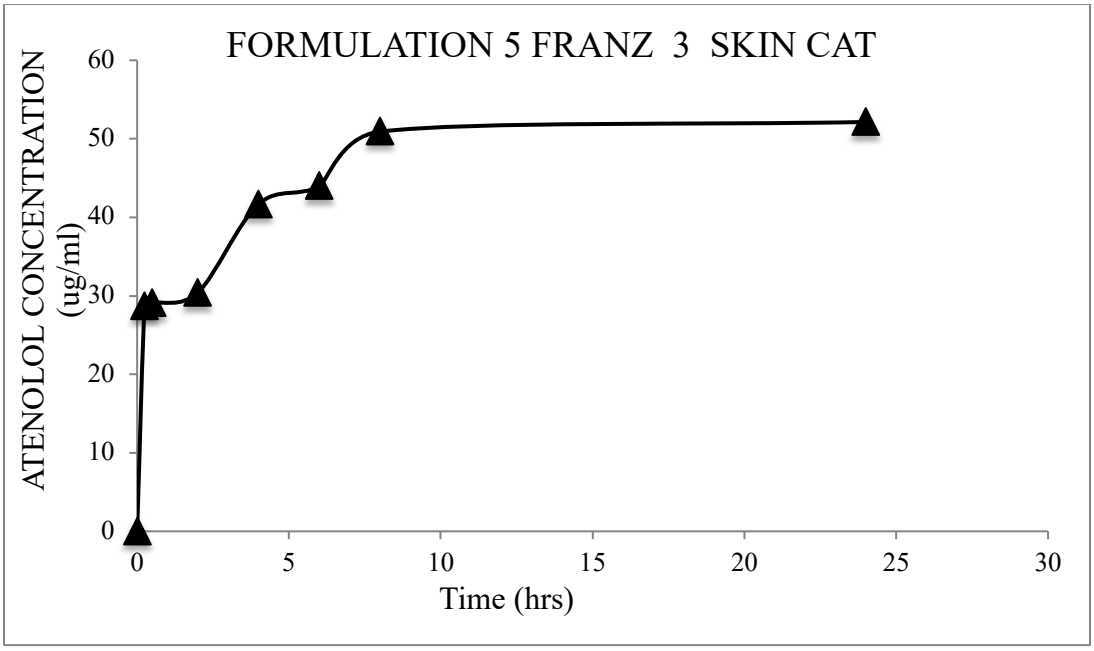

Figure A.26 Individual cat atenolol concentration time curve for formulation 5 through cat skin Franz 3

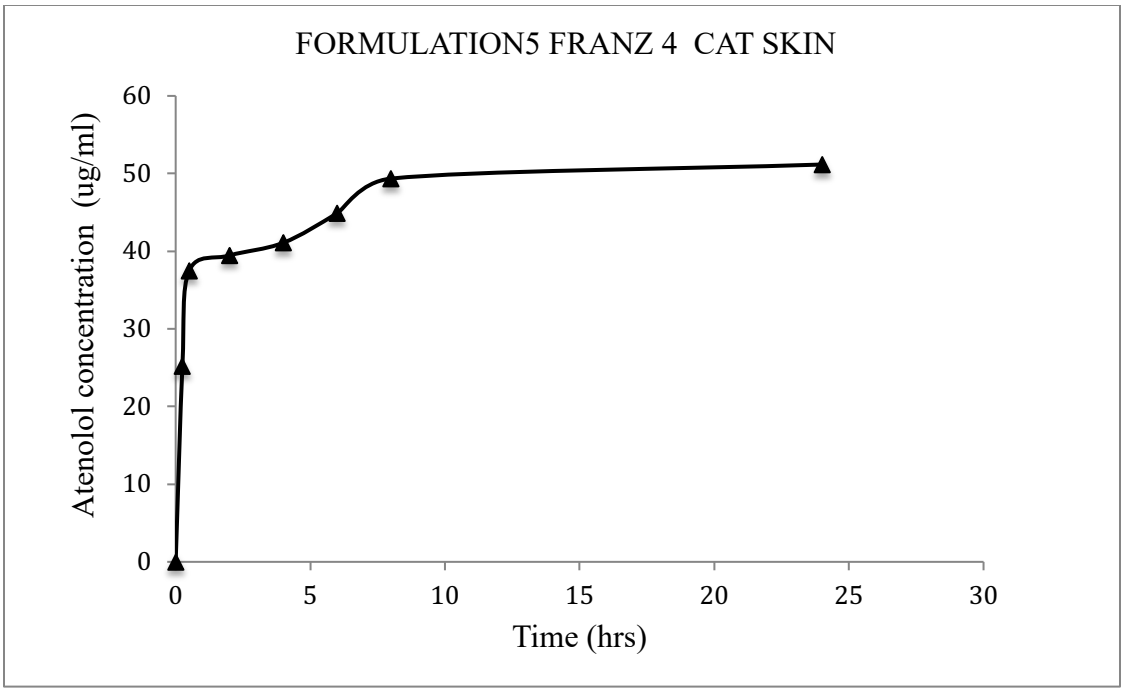

Figure A.27 Individual cat atenolol concentration time curve for formulation 5 through cat skin Franz 4.

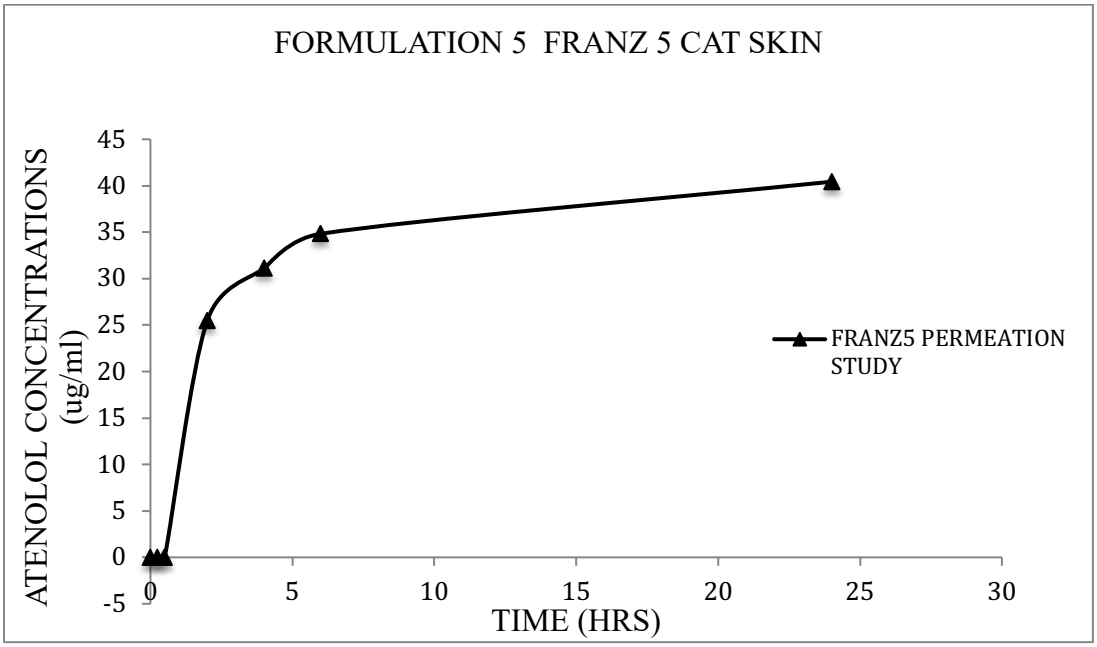

Figure A.28 Individual cat atenolol concentration time curve for formulation 5 through cat skin Franz 5.

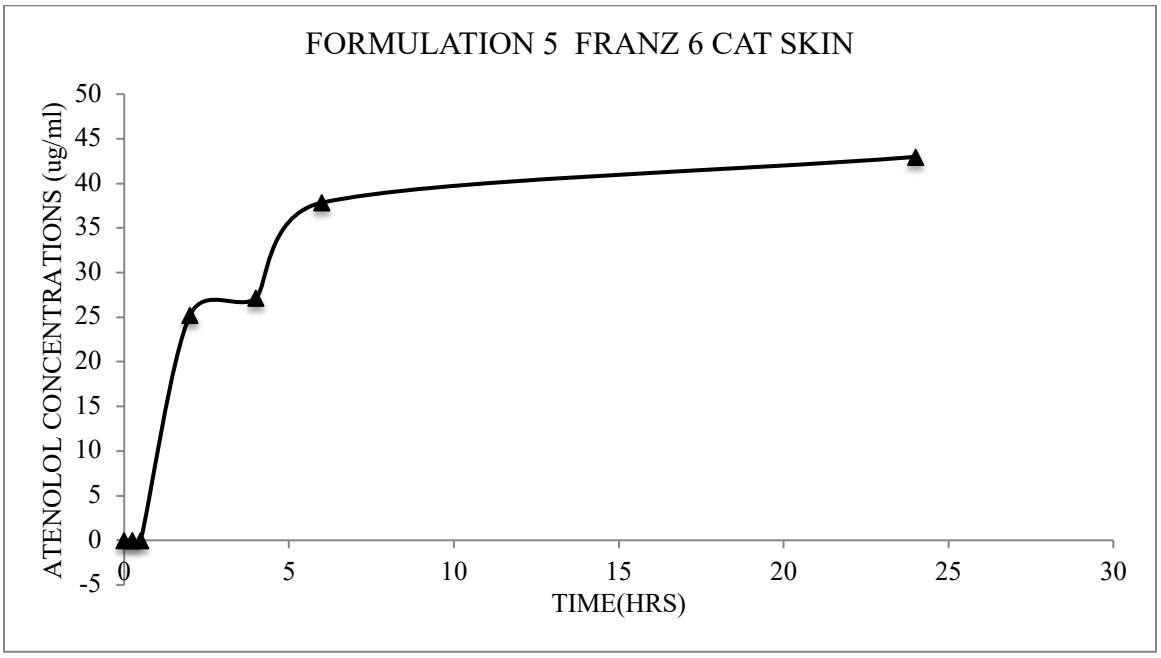

Figure A.29 individual cat atenolol concentration time curve for formulation 5 through cat skin Franz 6.

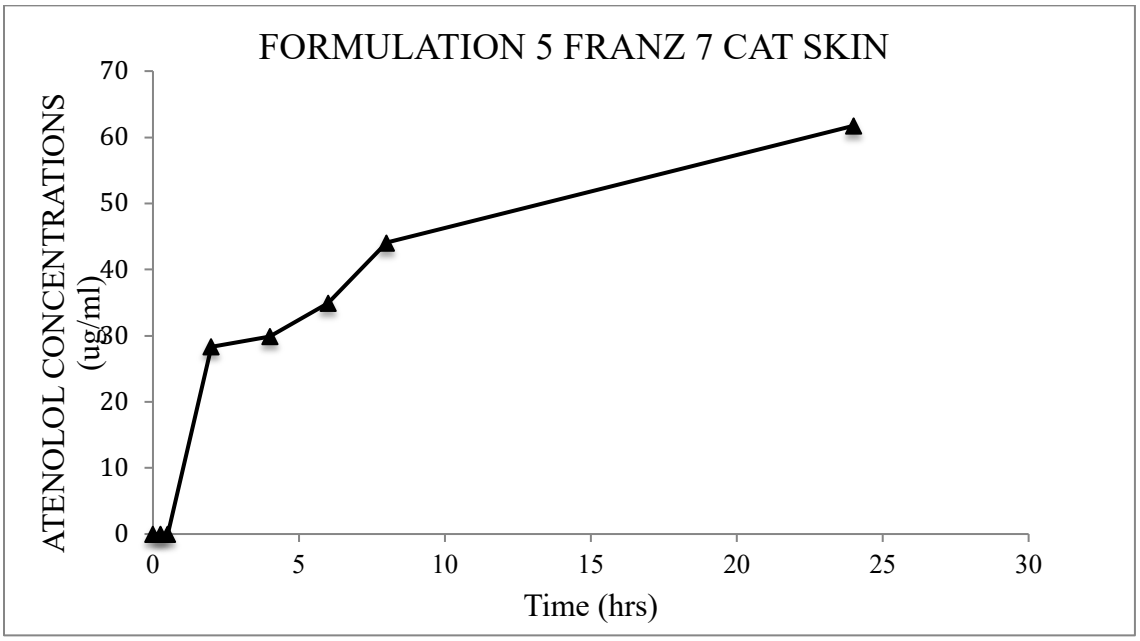

Figure A.30 Individual cat atenolol concentration time curve for formulation 5 through cat skin Franz 7

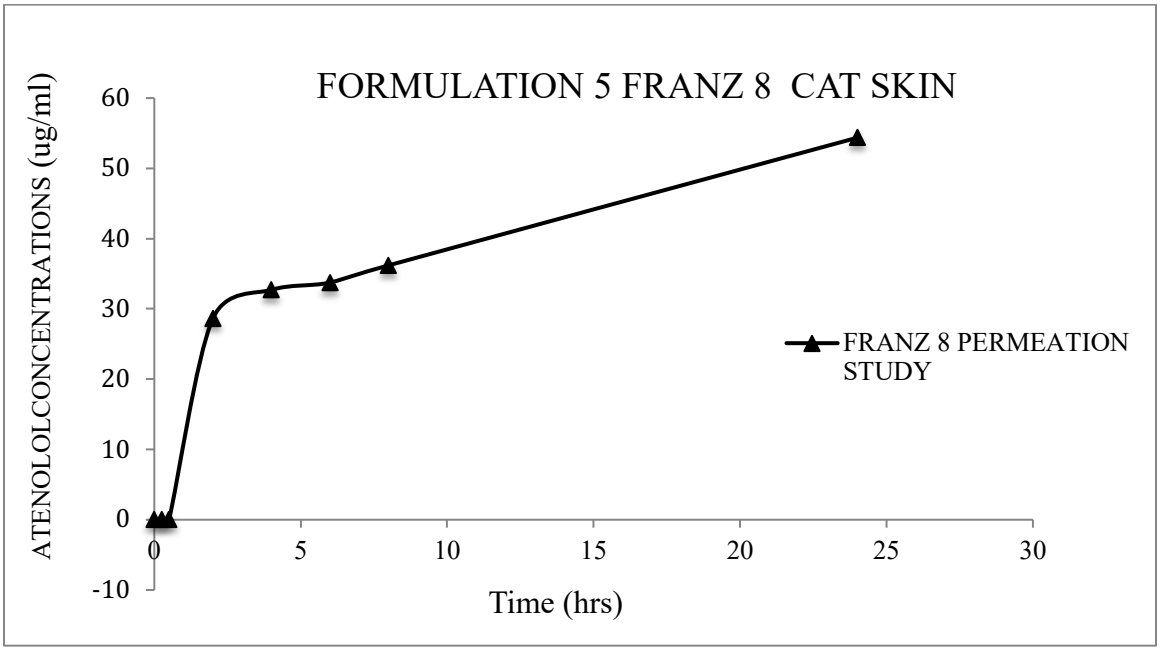

Figure A.31 Individual cat atenolol concentration time curve for formulation 5 through cat skin Franz 8

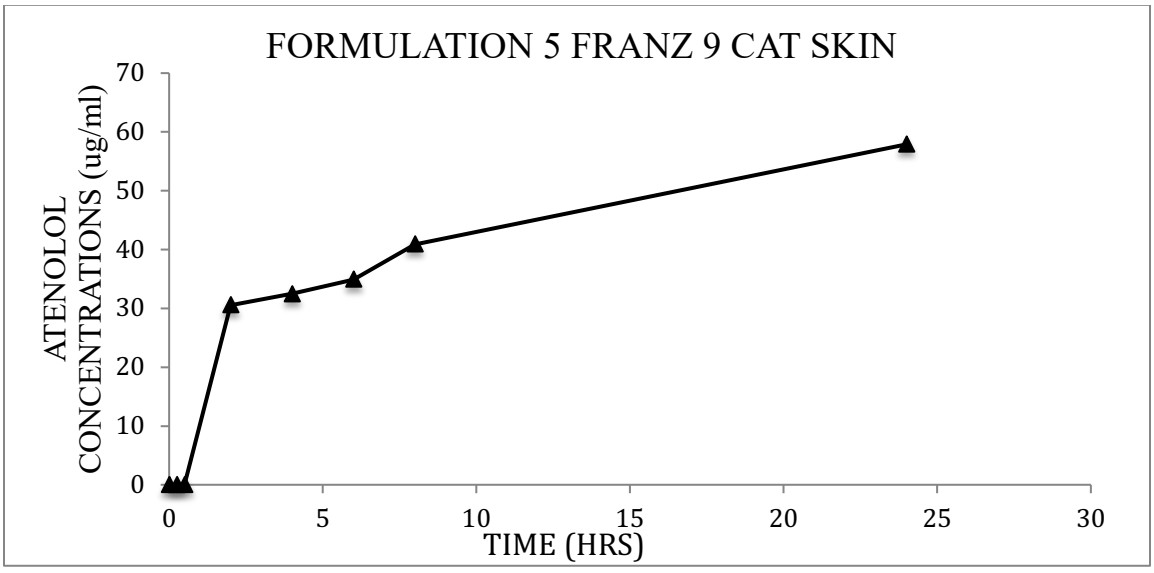

Figure A.32 Individual cat atenolol concentration time curve for formulation 5 through cat skin Franz 9.

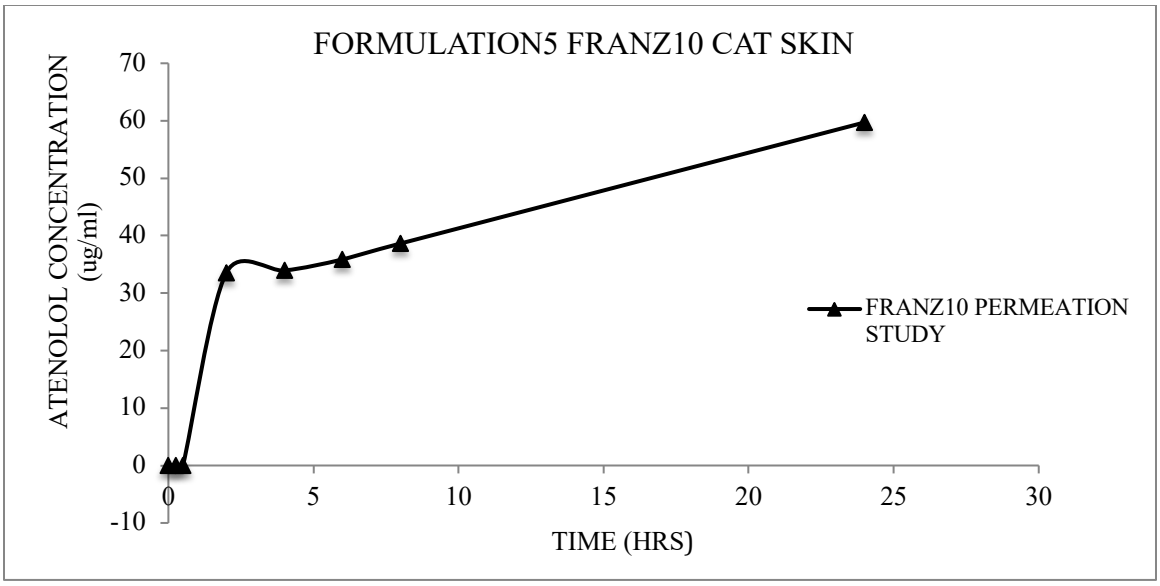

Figure A.33 Individual cat atenolol concentration time curve for formulation 5 through cat skin Franz 10**.**

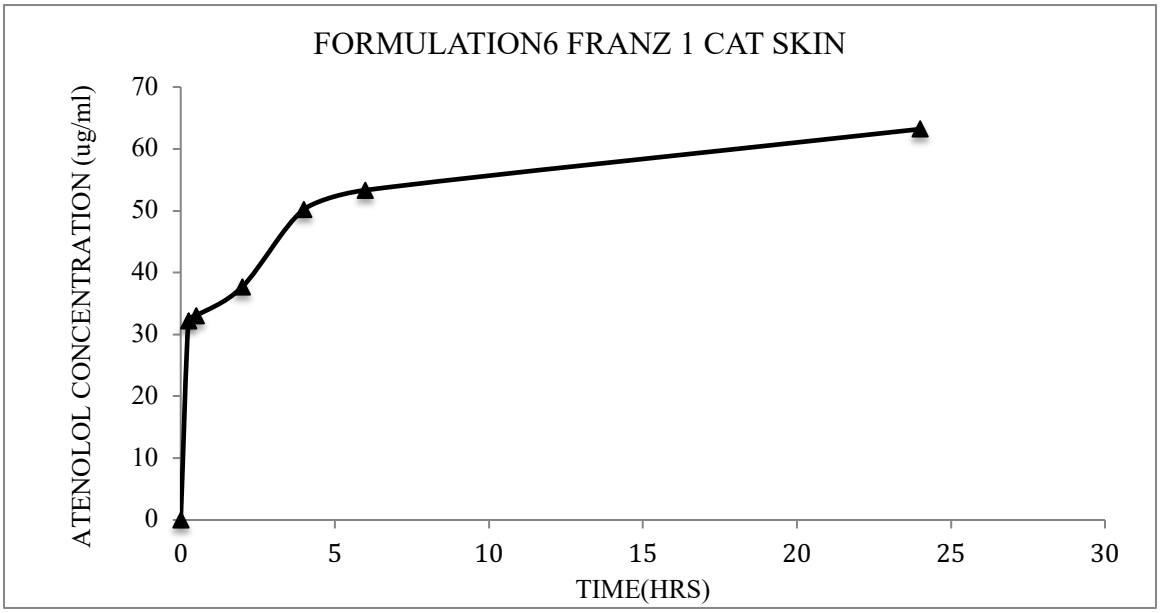

Figure A.34 Individual cat atenolol concentration time curve for formulation 6 through cat skin Franz 1.

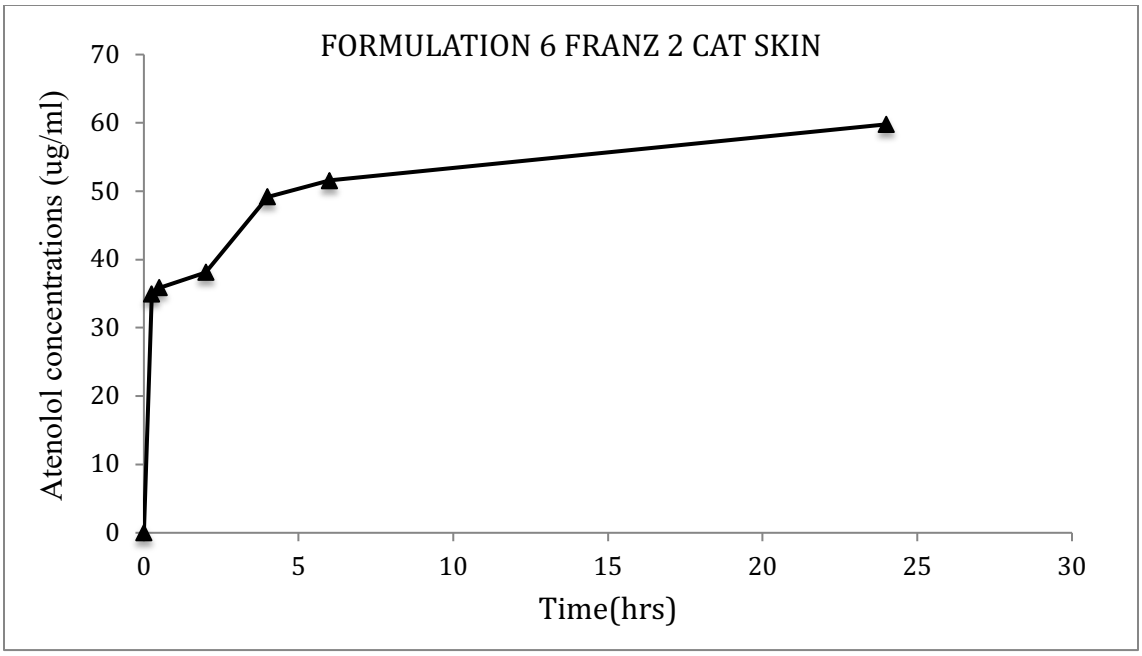

Figure A.35 individual cat atenolol concentration time curve for formulation 6 through cat skin Franz 2

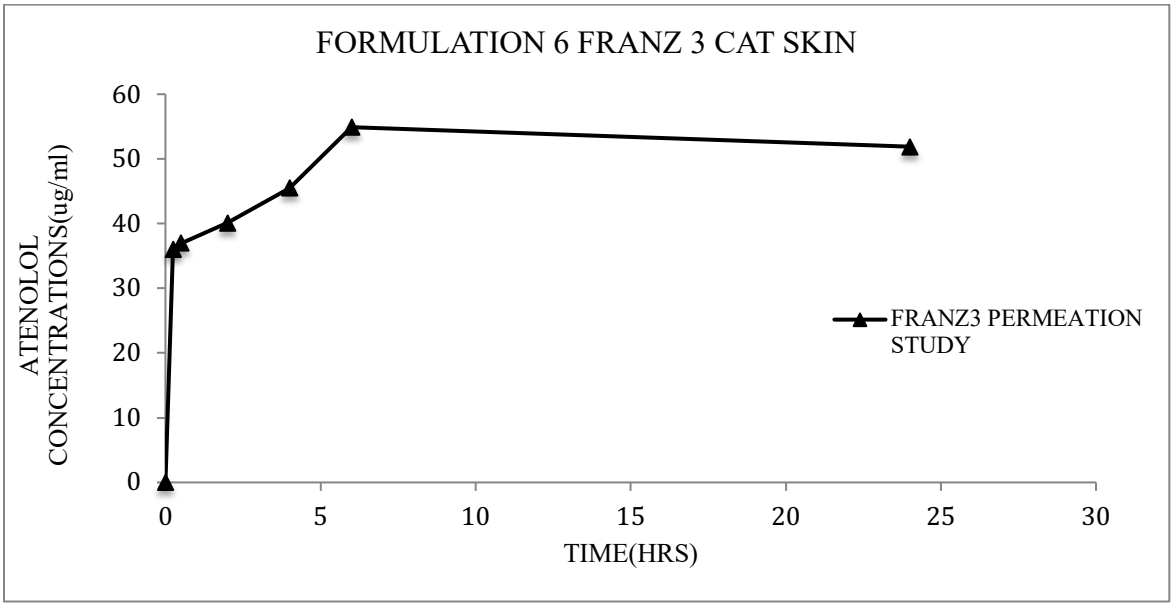

Figure A.36 Individual cat atenolol concentration time curve for formulation 6 through cat skin Franz 3.

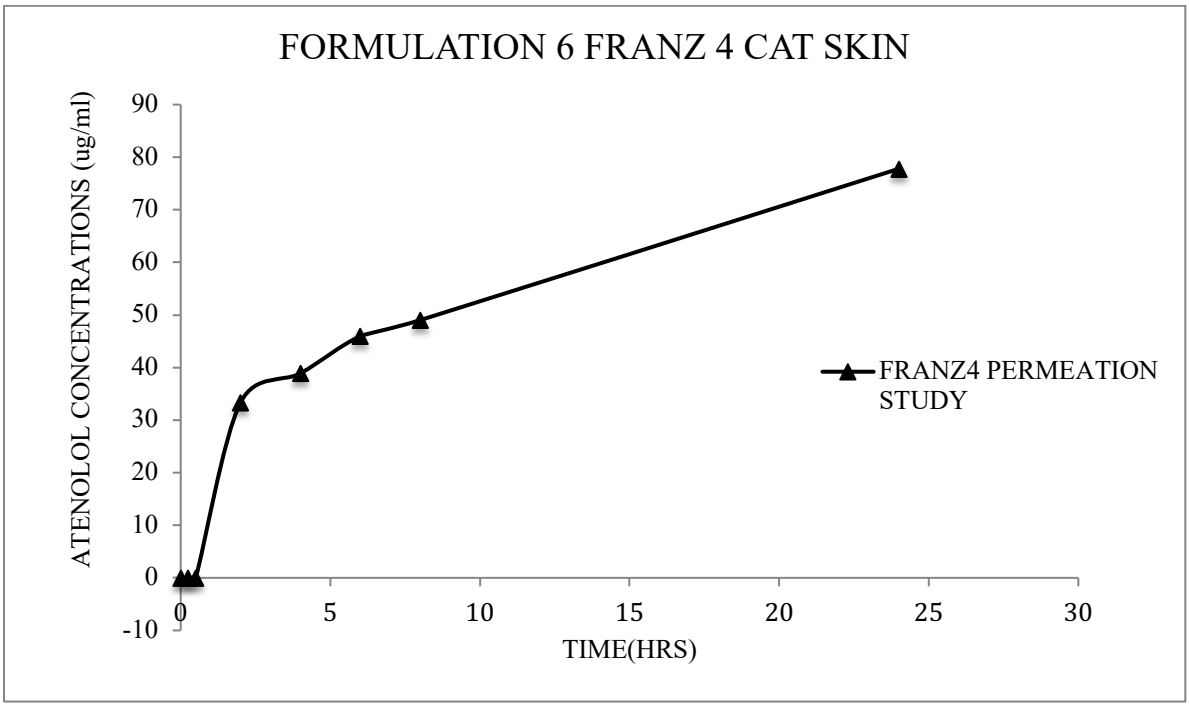

Figure A.37 Individual cat atenolol concentration time curve for formulation 6 through cat skin Franz 4.

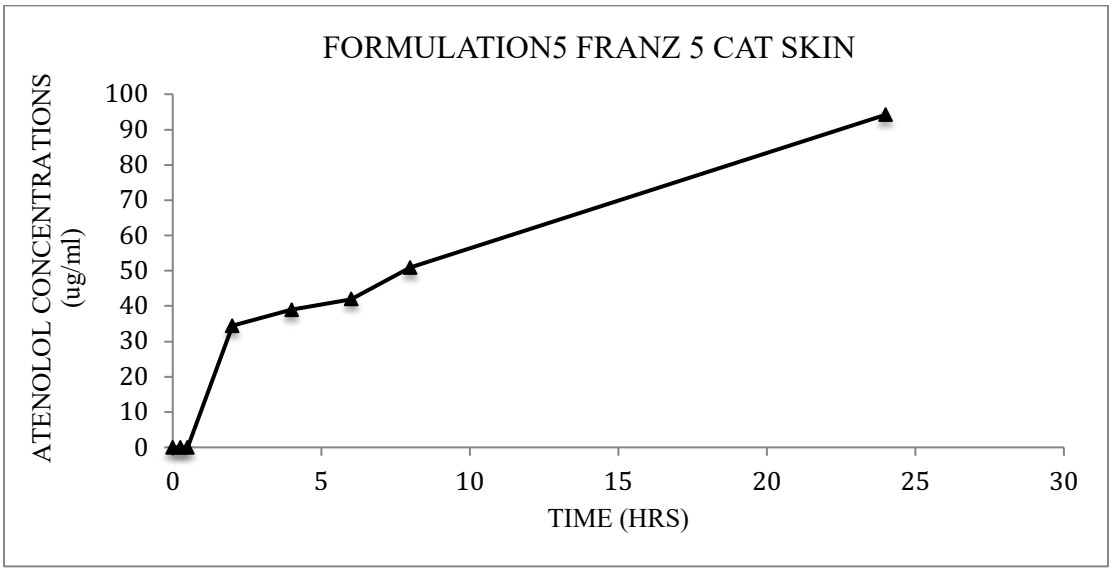

Figure A.38 Individual cat atenolol concentration time curve for formulation 6 through cat skin Franz 5.

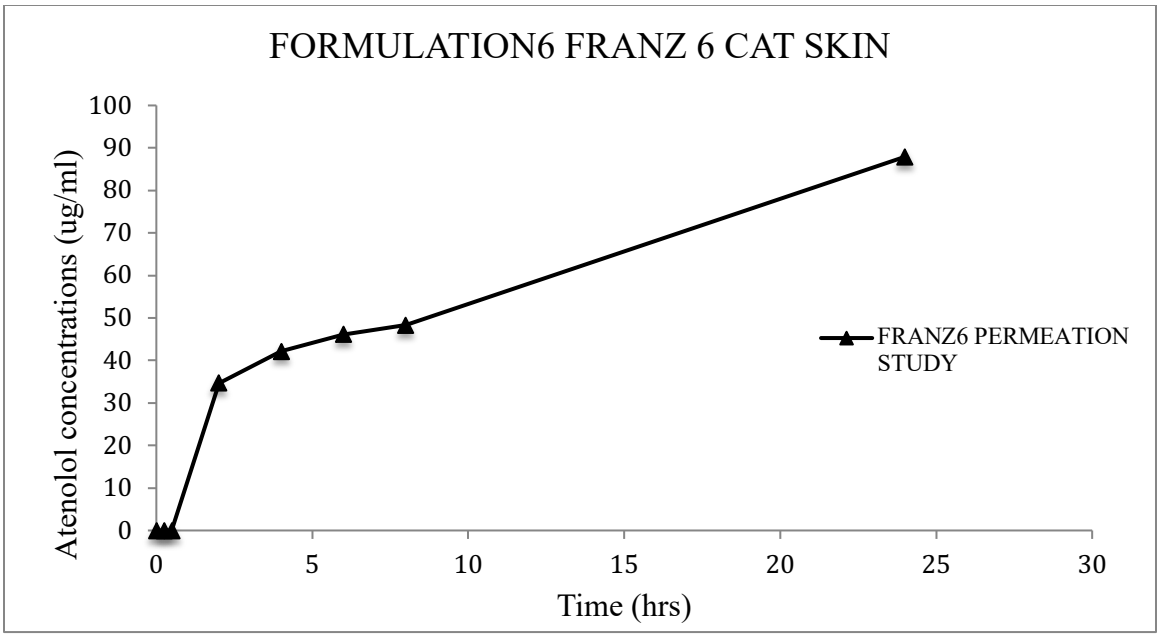

Figure A.39 Individual cat atenolol concentration curve for formulation 6 through cat skin Franz 6

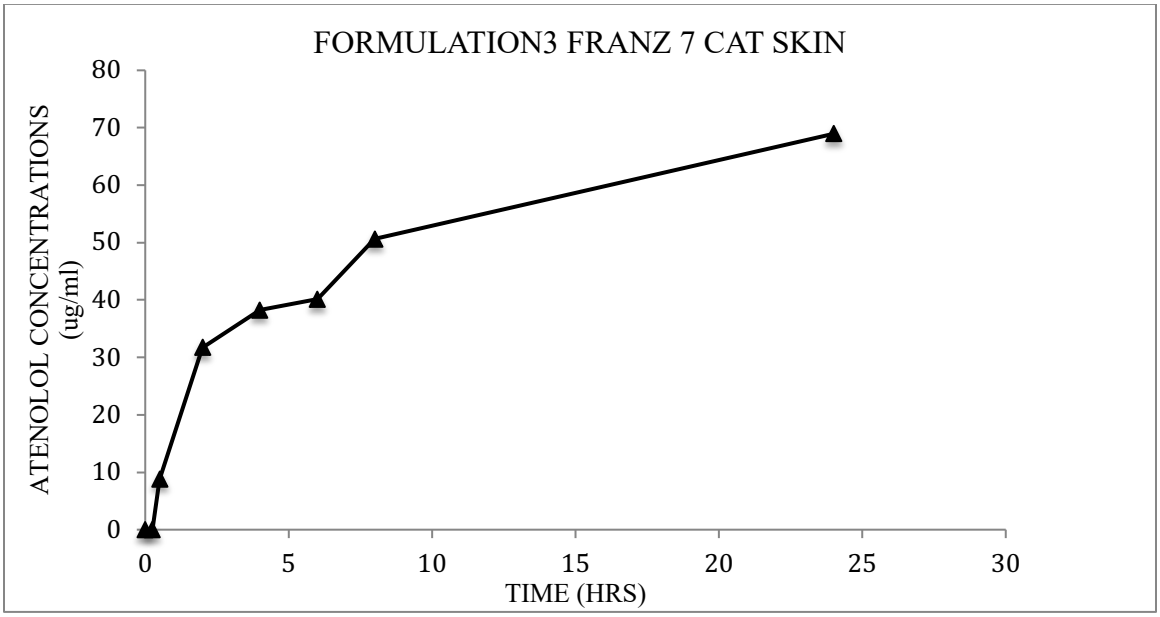

Figure A.40 Individual cat atenolol concentration time curve for formulation 6 through cat skin Franz 7

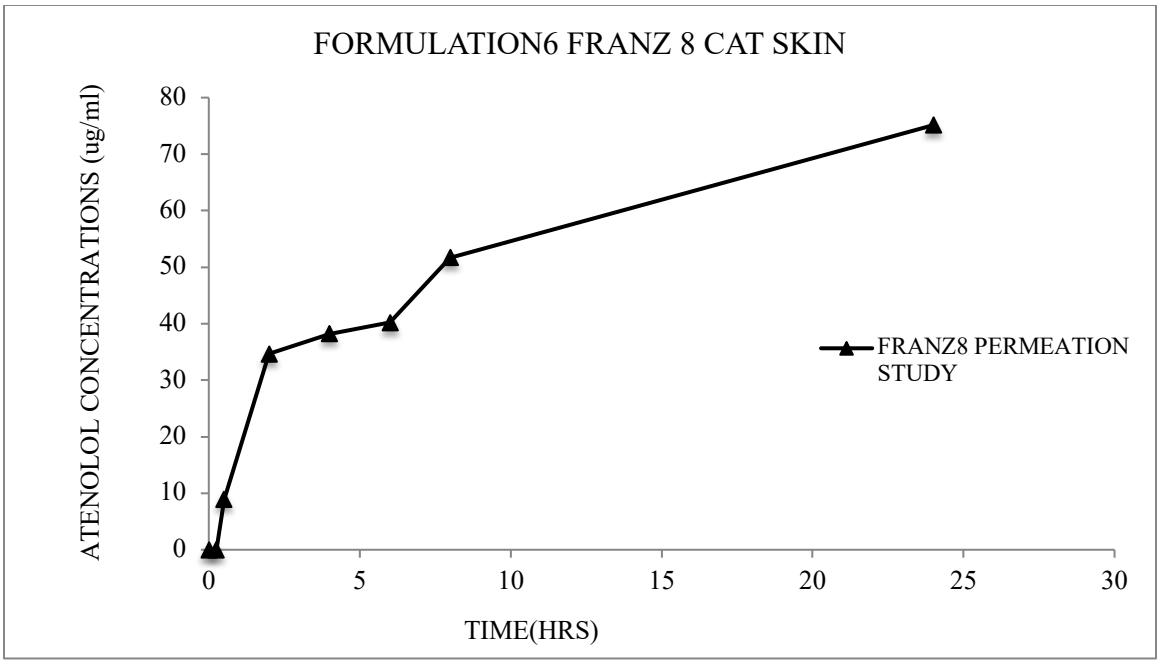

Figure A.41 Individual cat atenolol concentration curve for formulation 6 through cat skin Franz 8

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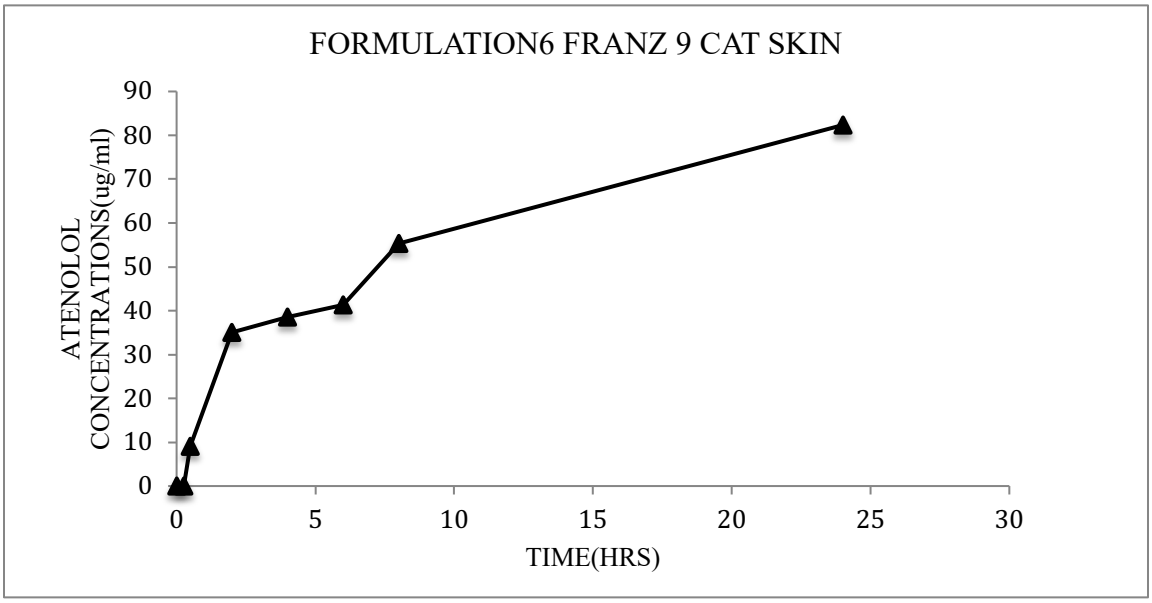

Figure A.42 Individual cat atenolol concentration time curve for formulation 6 through cat skin Franz 9.

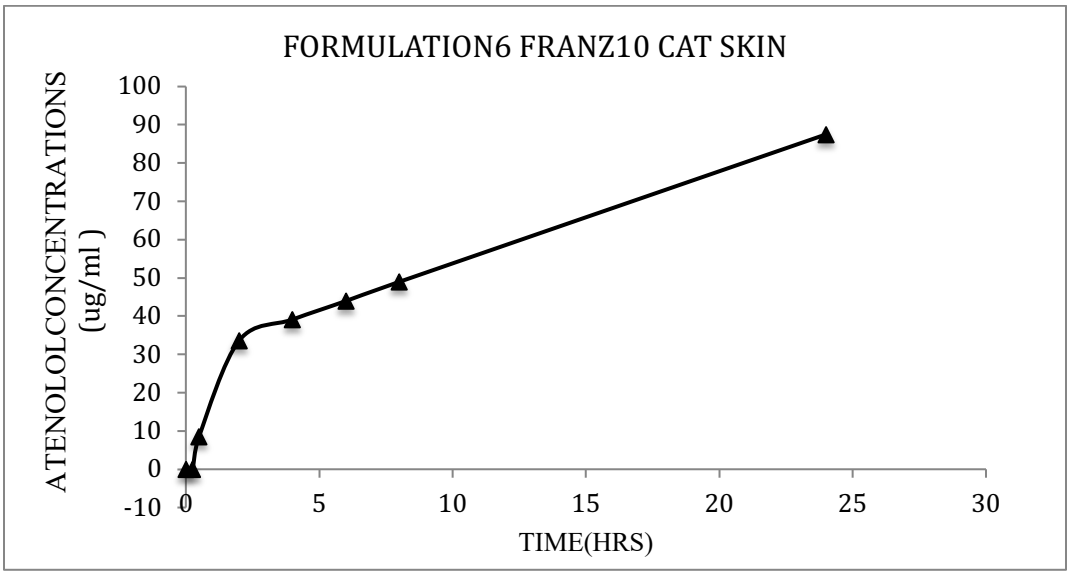

Figure A.43 Individual cat atenolol concentration time curve for formulation 6 through cat skin Franz 10

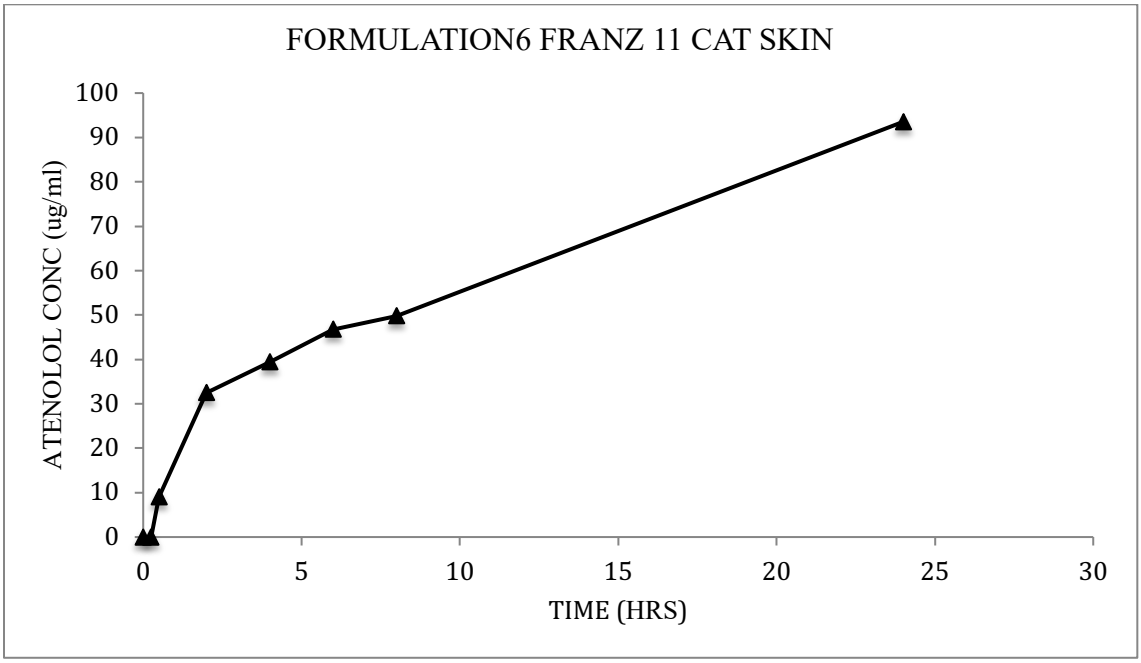

Figure A.44 Individual cat atenolol concentration time curve for formulation 6 through cat skin Franz 11.

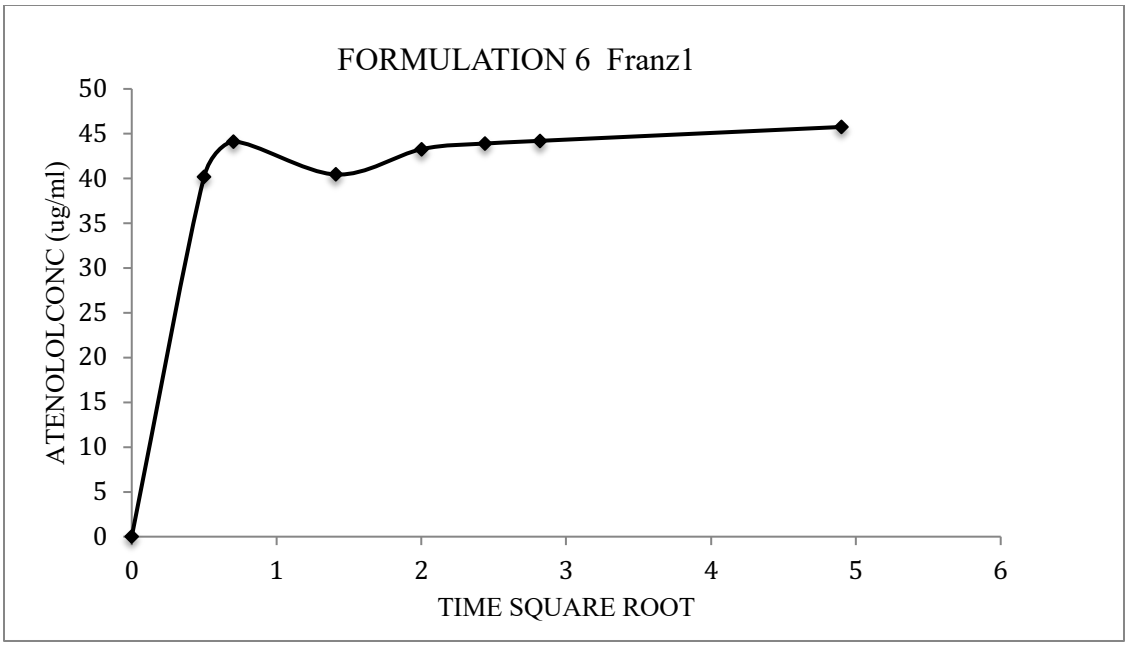

Figure A.45 Atenolol concentration versus square root versus plot for formulation 3 through cat skin Franz 1.

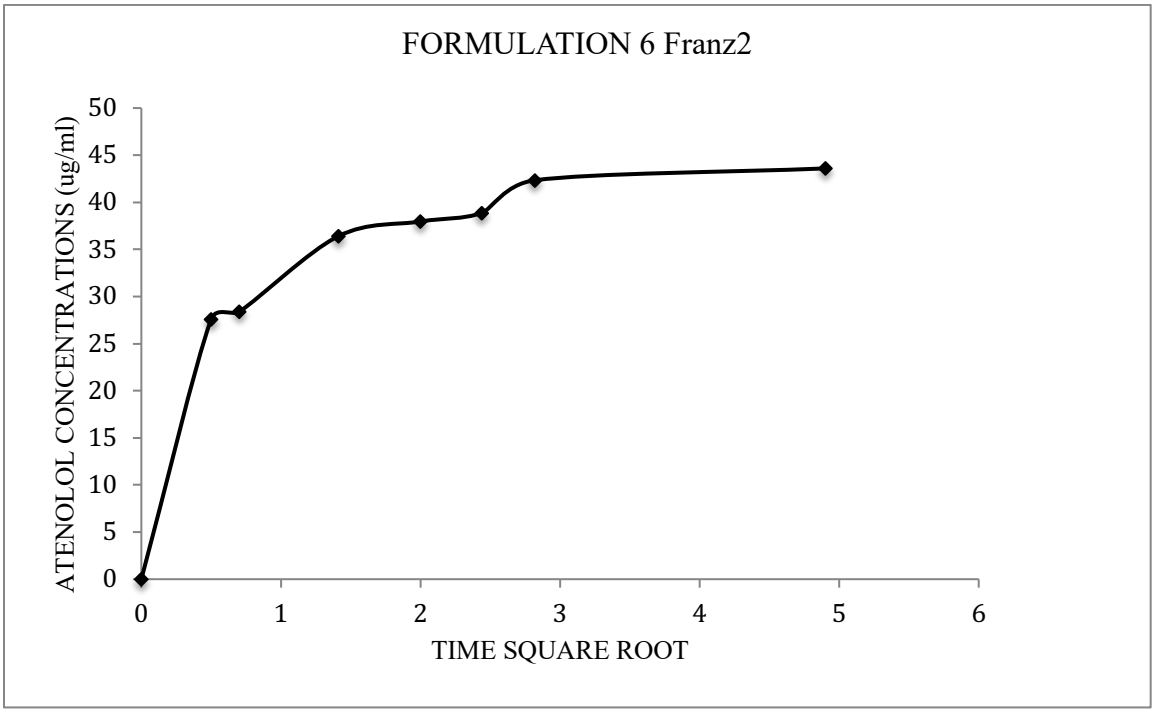

Figure A.46 Atenolol concentration versus square root of time plot for formulation 6 through cat skin Franz 2.

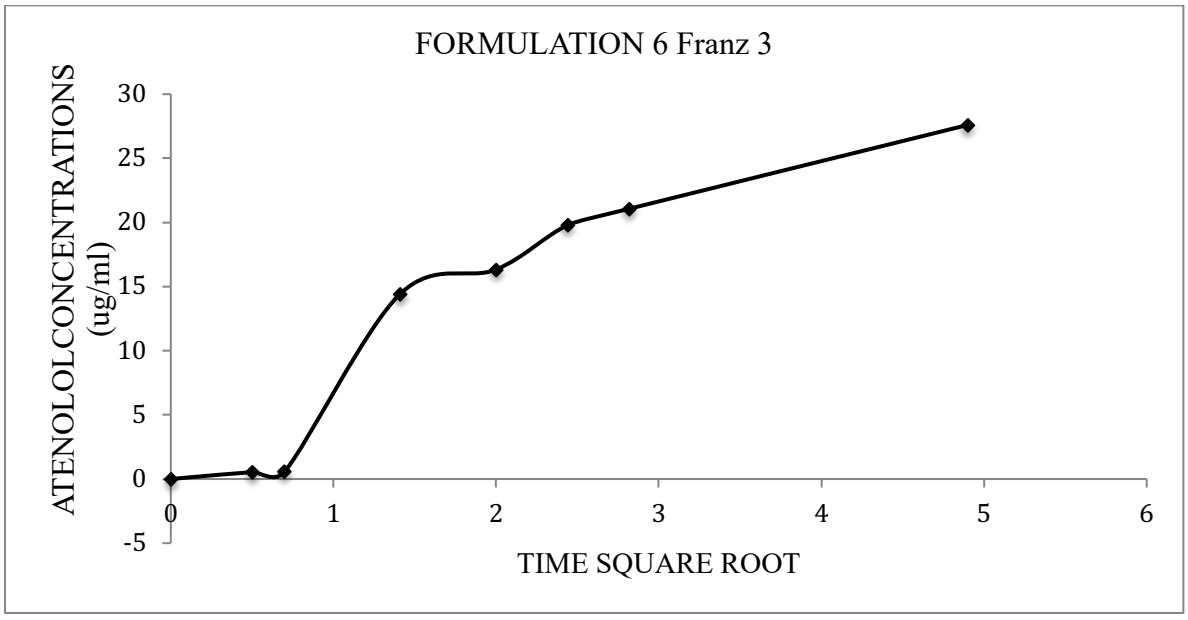

Figure A.47 Atenolol concentration versus square root of time for formulation 6 through cat skin Franz 3.

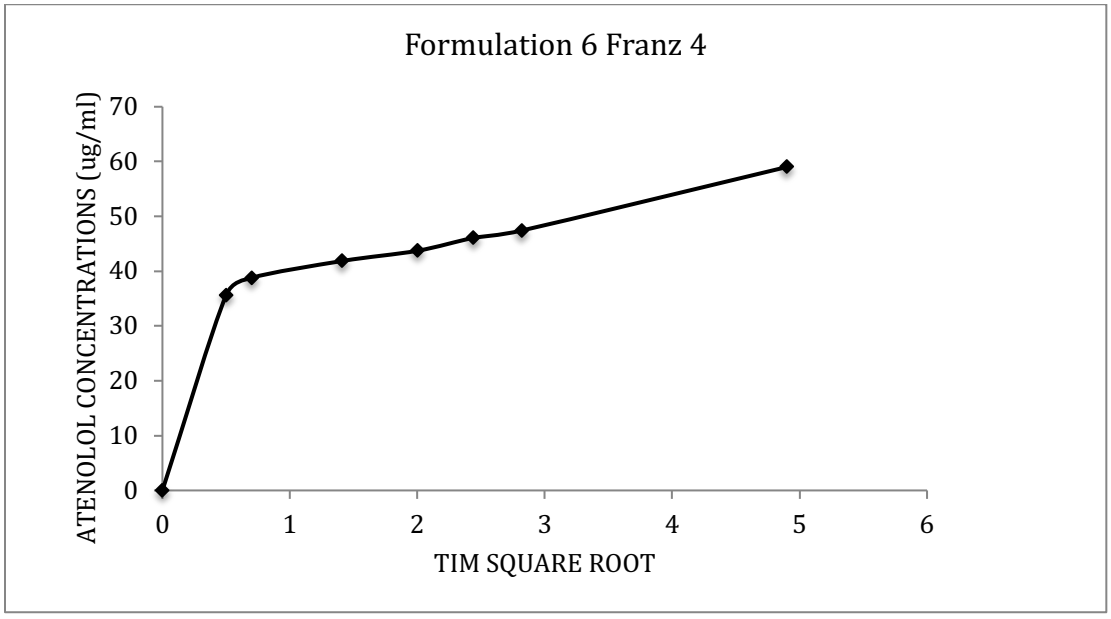

Figure A.48 Atenolol concentration versus square root of time plot for formulation 6 through cat skin Franz 4.

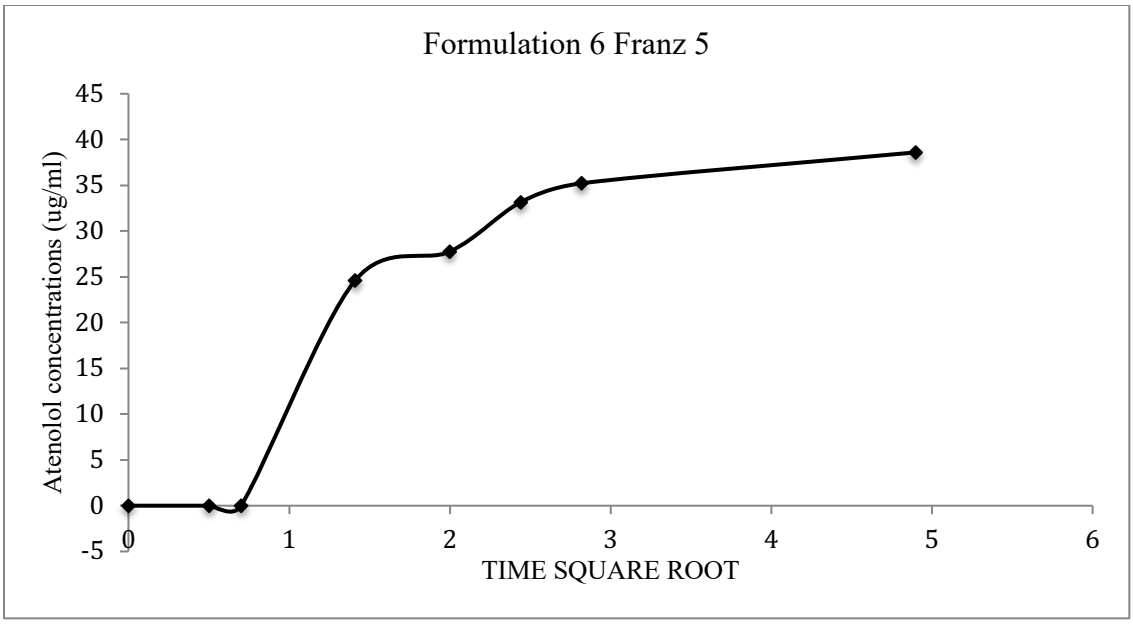

Figure A.49 Atenolol concentration versus square root of time plot for formulation 6 through cat skin Franz 5.

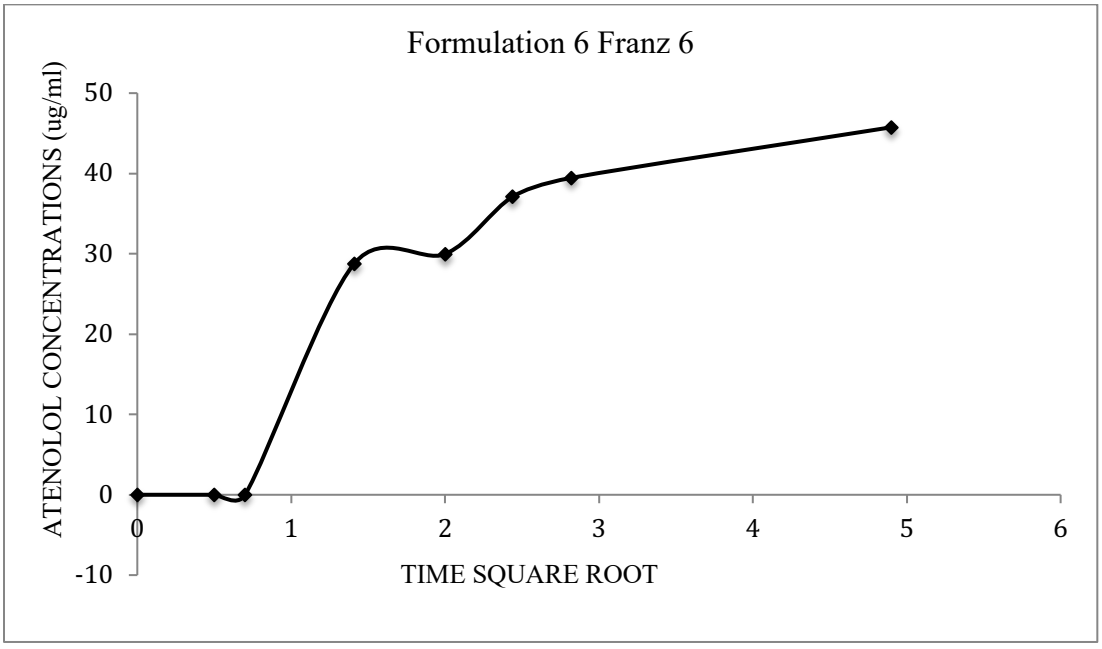

Figure A.50 Atenolol concentration versus square root of time plot for formulation 6 through cat skin Franz 6.

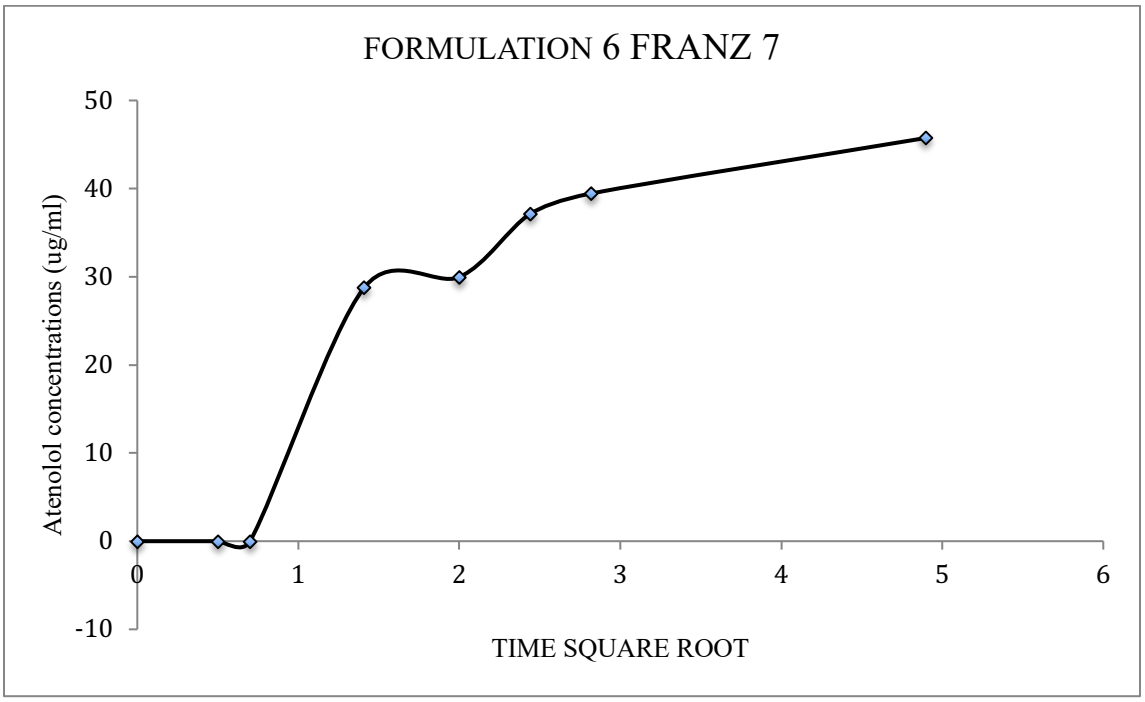

Figure A.51 Atenolol concentration versus square root of time plot for formulation 6 through cat skin Franz 7.

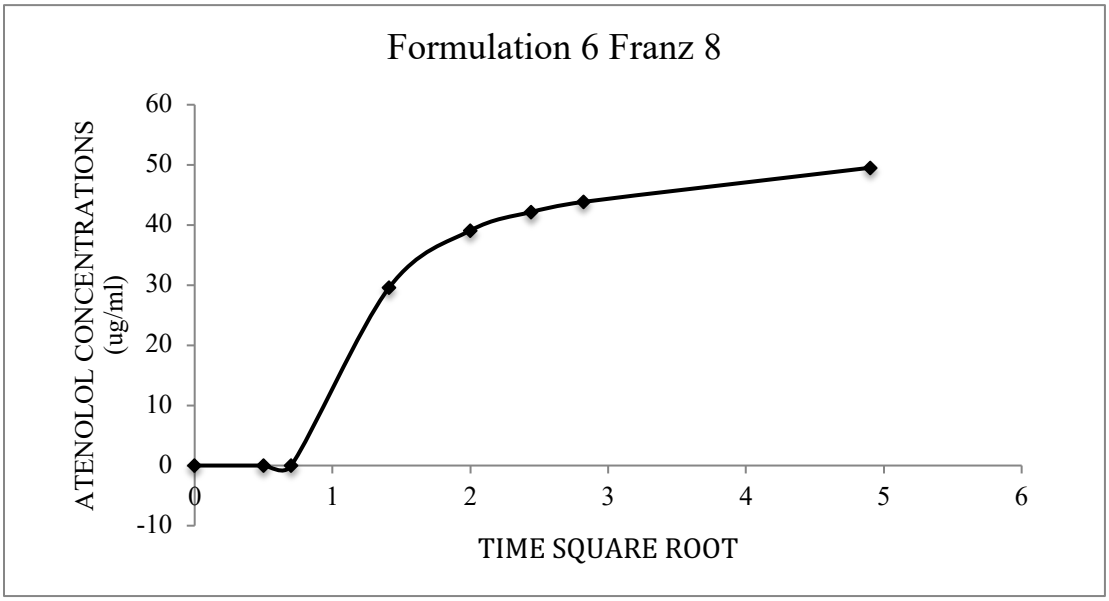

Figure A.52 Atenolol concentration versus square root of time plot for formulation 6 through cat skin Franz 8.

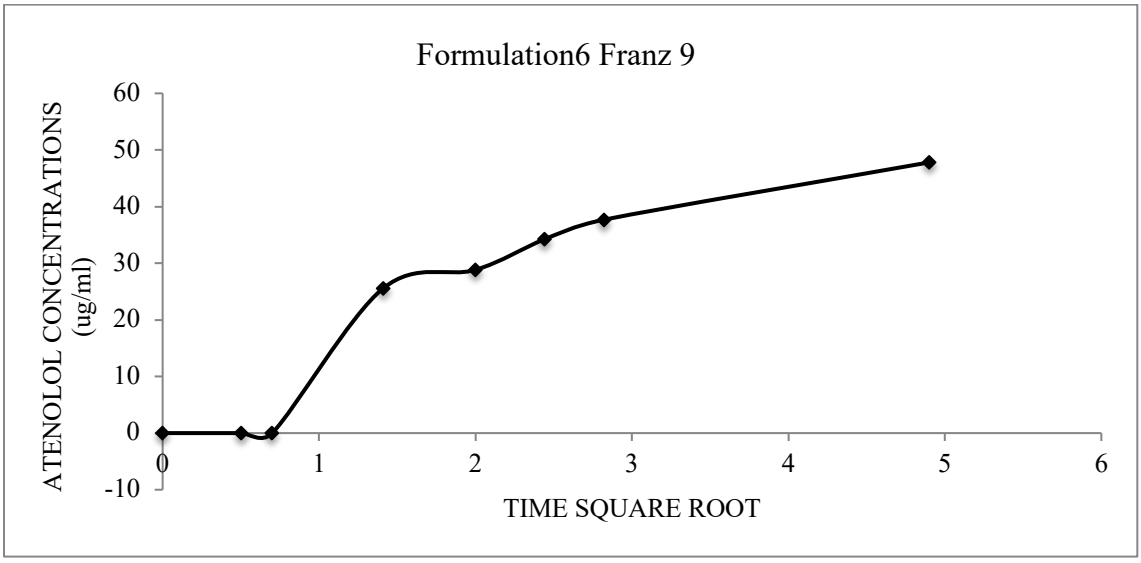

Figure A.53 Atenolol concentration versus square root of time plot for formulation 6 through cat skin Franz 9.