Chapter 10

IN VIVO

PHARMACODYNAMIC AND

PHARMACOKINETIC STUDY

10.1 Introduction

Pharmacokinetics is defined as the study of the time course of drug absorption, distribution, metabolism, and excretion. Clinical pharmacokinetics is the application of pharmacokinetic principles to the safe and effective therapeutic management of drugs in an individual patient. The development of strong correlations between drug concentrations and their pharmacologic responses has enabled clinicians to apply pharmacokinetic principles to actual patient situations. A drug's effect is often related to its concentration at the site of action, so it would be useful to monitor this concentration. [1] Pharmacodynamics refers to the relationship between drug concentration at the site of action and the resulting effect, including the time course and intensity of therapeutic and adverse effects. Thus, Pharmacokinetics is the discipline that applies mathematical models to describe and predict the time course of drug concentrations in body fluids, whereas pharmacodynamics refers to the time course and intensity of drug effects on the organism, whether human or experimental animal. [2]

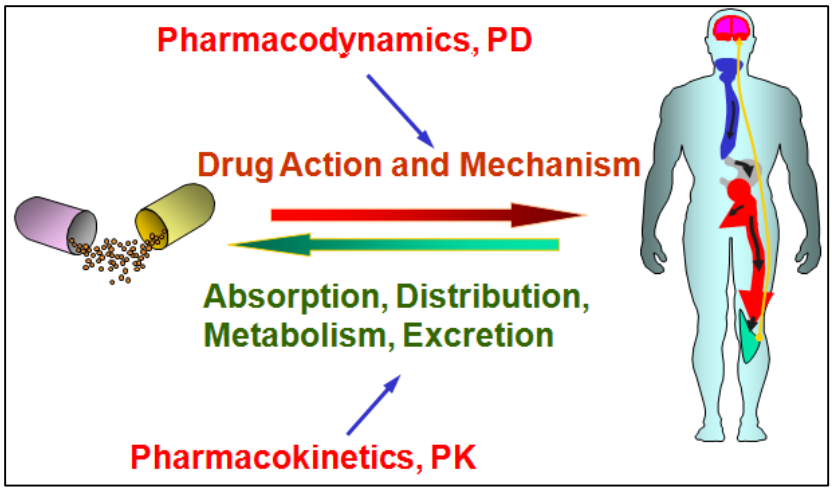


Figure 10.1 Relationship of Pharmacokinetics and Pharmacodynamics

The aim of the present study was to observe the effect of liposomal formulations on the uterine fibroids induced in rabbits and to compare the effect of three different formulations viz. LA-Liposomes, RLX-Liposomes and RLX-LA-Liposomes. The first step to diagnose the uterine fibroids and endometriosis is through ultrasonography. It uses sound waves to get an image of the uterus to confirm any irregularities in the shape and

size of uterine lumen and to map and measure fibroids. [3] The concentration of drug in plasma of rabbit at various time points was also monitored to ensure that the drug retained in uterus and there was no systemic absorption.

10.2 Materials and Equipment

Materials

Diethylstilbestrol (DES) was purchased from Sigma Aldrich, St. Louis, MO, USA. 22 Gauge needles, syringes, hair removal cream and olive oil were procured from local Pharmacy. Membrane filter (0.22 microns) was purchased from Pall Life sciences, USA. HPLC grade Choloroform, Methanol, Formic acid, Acetonitrile, Ammonium formate and Propionic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Millipore Milli-Q gradient purified water (Molsheim, France) was used throughout the study. Supelco solid extraction tubes were purchased from Sigma Aldrich, USA. All other chemicals and solvents were of analytical reagent grade and were used without further purification.

Equipment

- Ultrasonography Machine (MyLab30vetgold, Esaote, Italy)
- LCMS-MS (ekspert^{TM} ultraLC with ekspert^{TM} ultraLC 100 pump system (eksigent-AB Sciex, Framingham, MA) coupled with 3200 QTRAP mass spectrometer (AB Sciex, Framingham, MA)
- Vortex mixer (CM 101 Plus, Remi Lab. Instruments, India)
- Digital Analytical balance (ATX 224, Shimadzu, Japan)
- Cooling centrifuge (Remi Equipments, Mumbai, India)
- Ultrasonic Bath 120W (Vibronics Co. Pvt. Ltd., Mumbai, India)

10.3 Pharmacodynamic Study

10.3.1 Induction of Uterine Fibroids in Rabbits

All animal experiments conducted were approved by the Institutional Animals Ethics Committee of the Institute of Pharmacy Department, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. New Zealand white female rabbits (aged 8-10 week), weighing between 1.5 to 2.5 kgs maintained on normal diet, were procured from Animal Vaccine Insitute, Gandhinagar, Gujarat, India.

Five female rabbits, 2 in each separate cages were maintained on green leafy vegetables and under controlled light (12 hour light: 12 hour dark) at ambient temperature of 22-25°C. One rabbit each was assigned to following 5 groups:
Group I: Normal/ Negative Control
Group II: Fibroid model/Positive Control
Group III: Liposomal Leuprolide acetate treated (0.308 mg)
Group IV: Liposomal Raloxifene Hydrochloride treated (6 mg)
Group V: Dual drug entrapped (RLX-LA) Liposomal formulation treated

The main objective of this study was to establish the effectiveness of the developed formulations on the uterine tumors. Since the formulations were administered by intravaginal route, which is a new route to achieve drug targeting to uterus, Pharmacodynamic study provides a strong basis to the research work.

Ultrasonography (MyLab30vetgold, Esaote, Italy) of uterus of all the animals was first done to observe the normal uterine structure. Images were recorded for each group. The procedure was carried out at Veterinary Polyclinic, Vadodara, India. Before the ultrasonography, rabbits were shaved on the dorsal side to remove the fur near the vagina and uterus. This was done to allow the probe of sonography machine to function properly and avoid any hindrance due to furs, in the procedure. Figure 10.2 shows the rabbits with dorsal side shaved and fur removed with the help of hair removal cream.

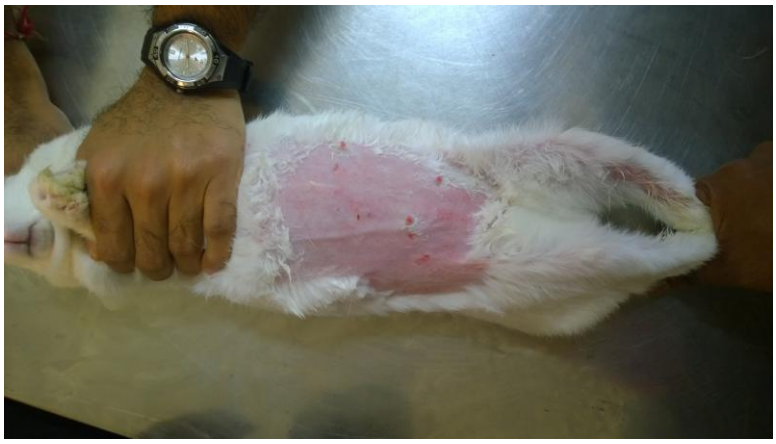


Figure 10.2 Rabbit with dorsal side shaved for ultrasonography

10.3.2 Tumor regression analysis by Ultrasonography

Uterine Fibroid was induced in animals from group II-V by injecting Diethylstilbestrol subcutaneously. [4, 5, 6, 7] Briefly, 20 mg of DES was weighed in four eppendorf tubes each. 2-2.5 ml of sterilized (passing through 0.22 microns membrane filters, Pall Life Sciences) olive oil was added to each tube containing DES. The mixture was stirred on a vortex mixer for 5 minutes to dissolve the DES in the oil. It was then kept in bath sonicator for 2-3 minutes for deaeration. The solutions were then filled in separate 5 ml syringes with 22-gauge needles. Rabbits from Group II-V were injected with this solution subcutaneously. The same procedure was repeated weekly till the tumor formation in uterus begins which usually takes around 1.5-2 months. Every week ultrasonography of the rabbits was done to assess the tumor formation.

After the ultrasonography test confirms the formation of fibroids within the uterine cavity, the rabbits were given specific treatment according to their groups. Group III was treated with Liposomal Leuprolide acetate, Group IV with Liposomal Raloxifene Hydrochloride while Group V was administered with Liposomal formulation having dual drugs (LA-RLX). The Intravaginal rods were prepared for each formulation as per the method described in chapter 5, section 5.6. The rod was inserted in to the vaginal tract of rabbit with the help of vaginal insert applicator under the supervision of a veterinarian. Ultrasonography of all the rabbits with rod inserts was done every week to monitor the

tumor regression. Images were recorded for each rabbit. Figure 10.3 shows the ultrasonography procedure of the rabbits.

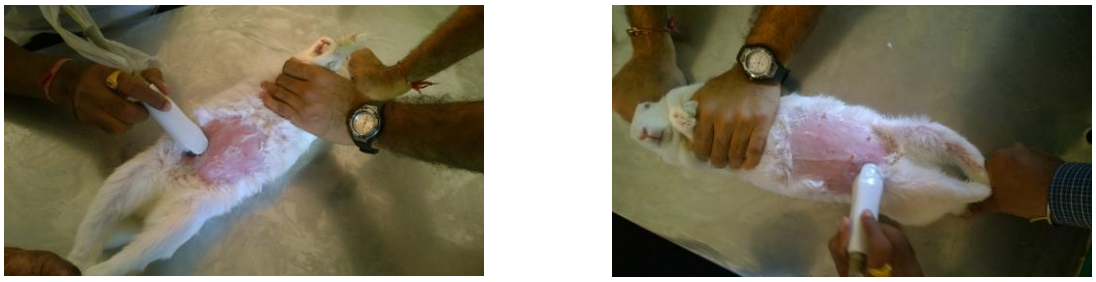


Figure 10.3 Ultrasonography procedure of the rabbits

10.4 Pharmacokinetic Study

All animal experiments conducted were approved by the Institutional Animals Ethics Committee of the Institute of Pharmacy Department, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. New Zealand white female rabbits (aged 8-10 weeks), weighing between 1.5 to 2.5 kgs maintained on normal diet, were procured from Animal Vaccine Insitute, Gandhinagar, Gujarat, India.

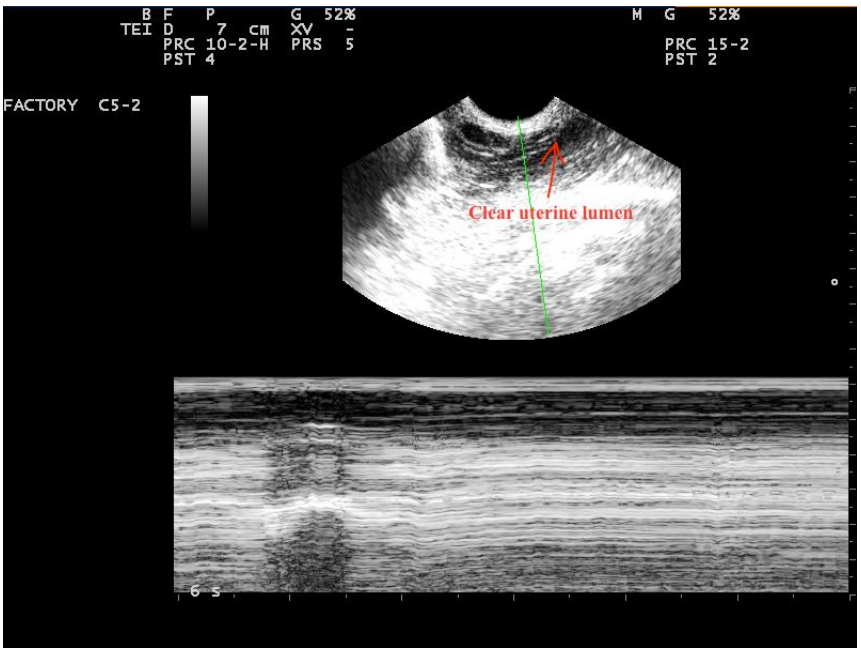
The main purpose of this study was to monitor the systemic absorption of the drugs if any, from the liposomal formulation loaded intravaginal rods in New Zealand white female rabbits of the group III and IV of the Pharmacodynamic study. After the intravaginal rod insertion containing liposomal LA and RLX separately in one rabbit each, about 0.5 ml blood samples were withdrawn from the dorsal vein of the other ear of both the rabbits, at different time intervals (pre dose, 15 min, 30 min, 1 hour, 2 hours, 4 hours, 18 hours and 24 hours) and collected into pre-labeled heparinized eppendorf tubes. Plasma separation was carried out by centrifugation at 4000rpm for 5min at 25^{o}C. The analysis of concentration of LA and RLX in plasma at various time intervals was done using LCMS-MS. Plasma samples were treated in similar manner as described in sections 3.3.5 and 3.4.5 for the estimation of LA and RLX respectively.

10.5 Results and Discussion

The pharmaceutical industry is challenged by demand for innovative and highly efficacious medications, transforming its research and development operations to meet an ever-growing demand for more and more affordable drugs in highly competitive market in a shorter time period. A more rigorous in vivo study in the formulation development is thus required to establish the efficacy of the products. One of the most challenging steps in drug development is the choice of an appropriate dose range for early phase I studies. Pharmacokinetic and Pharmacodynamic concepts might be helpful in extrapolating preclinical data from animal species to humans, thereby facilitating dose escalation selection. Preclinical data extrapolated in vivo animal models serves as a reliable guide to identify a safe and effective dose and therapeutic concentration range for phase I clinical studies. [8]

10.5.1 Results of Pharmacodynamic studies

Figure 10.4 shows the ultrasonography images of the rabbits with normal uterus. As seen in the figure, the uterus shows clear lumen without any obstruction. This serves as negative control.



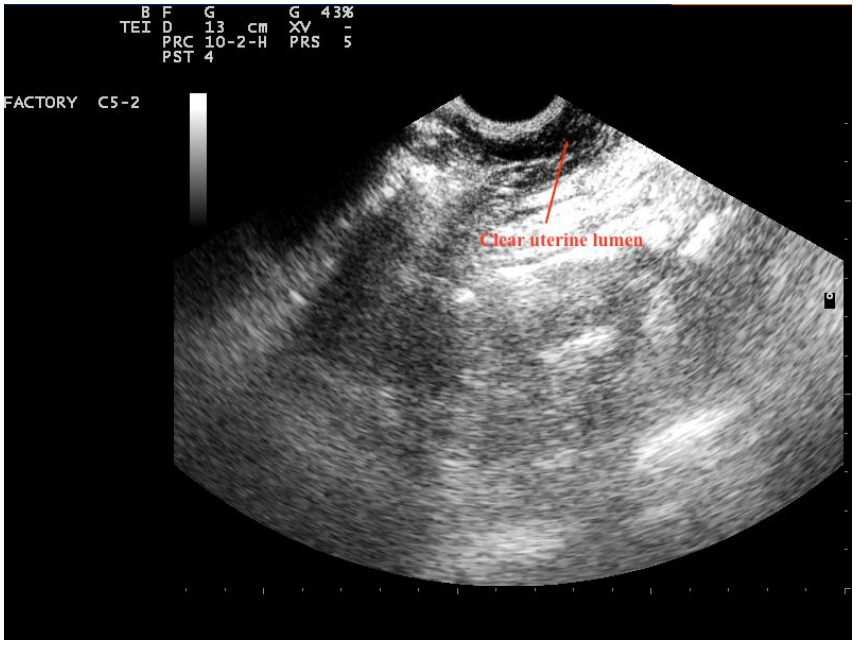
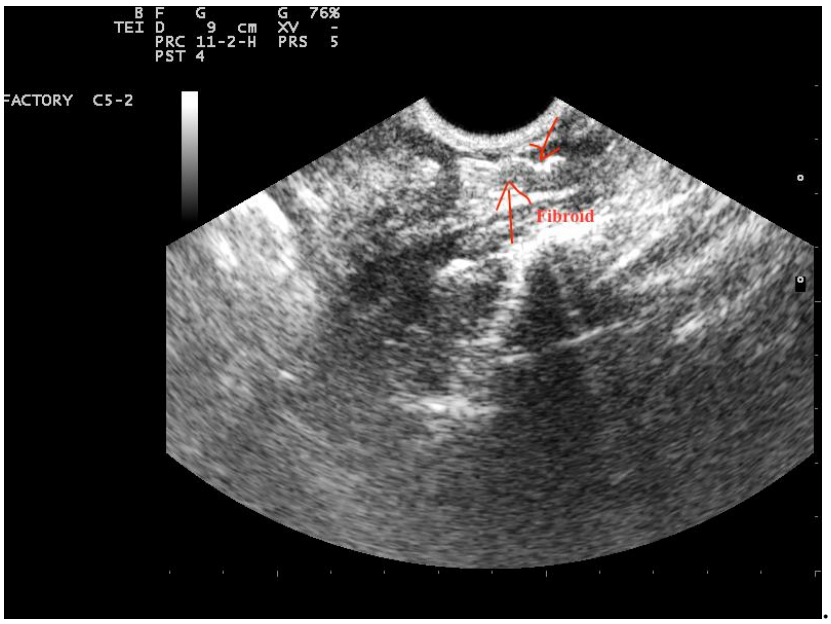


Figure 10.4 Ultrasonography of rabbit uterus depicting clear uterine lumen (Normal/Negative control)

Figure 10.5 reveals the tumor formation in the uterine lumen. The lumen shows obstruction due to fibroids growing from the endometrium of uterus. This serves as positive control. In the present study 80 mg of DES (oil based injection) for each rabbit was administered subcutaneously in divided doses of 20 mg every week, for four weeks.



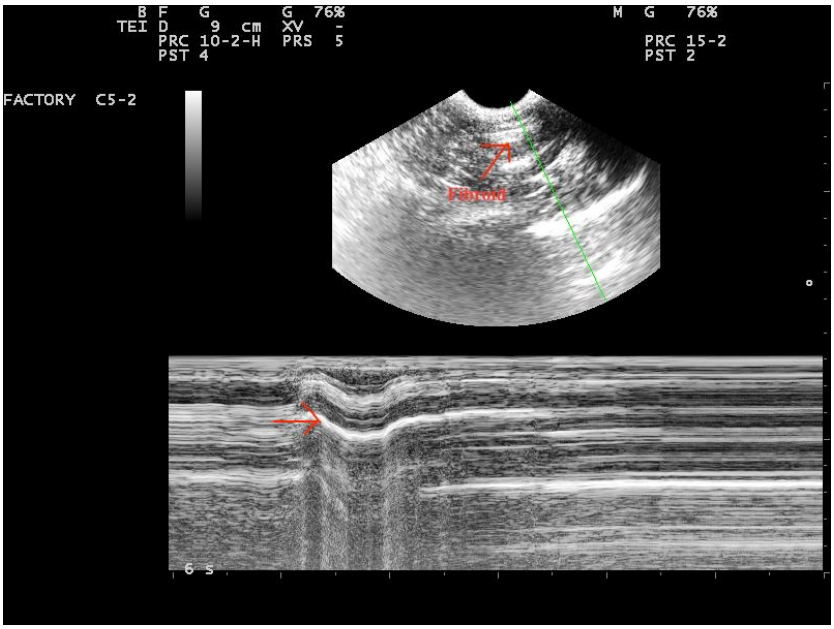
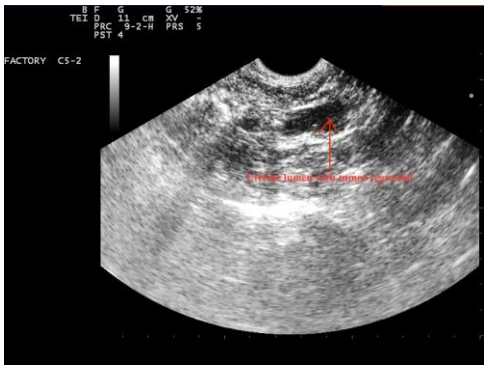


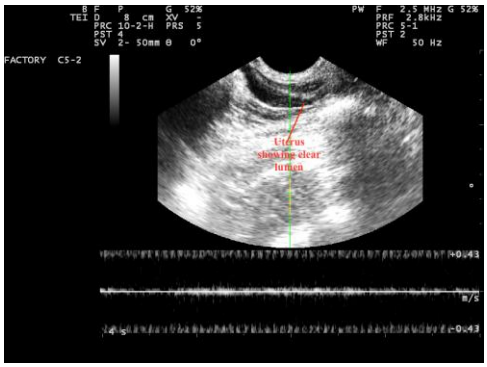
Figure 10.5 Ultrasonography of rabbit uterus showing uterine fibroid in the lumen (Positive control)

After the initiation of treatment with various liposomal formulations, ultrasonography of rabbits from group III-V was done every week to monitor the regression of tumors. The results showed that, noticeable fibroid regression started after 15 days of the formulation loaded intravaginal rod insertion into the vaginal cavity of rabbits. The images given in Figure 10.6 depict ultrasonography results of rabbits treated with different liposomal formulations. Rabbit, which was administered with liposomal formulation of Leuprolide acetate, showed better tumor regression than the rabbit with Raloxifene loaded liposomal formulation as seen in the Figure 10.6. The uterine lumen in the case of RLX-liposomal formulation treated rabbit though shows the regression effect but the lumen is not as clear off the fibroids as in the case of LA-liposomal formulation treatment. After 30 days of the initiation of the treatment, rabbit administered with RLX-LA (dual drug loaded) liposomal formulation shows highest tumor regression effect than the individual formulations. The results can be very well correlated with the findings of cell line studies, which showed maximum cell inhibitory effect, and highest DNA fragmentation (apoptosis) count in the RLX-LA liposomal formulation treated MCF-7 cells. Since the uterine leiomyoma has the presence of both GnRH as well as Estrogen receptors, both RLX and LA exhibit synergistic effect by acting on the respective receptors and thereby

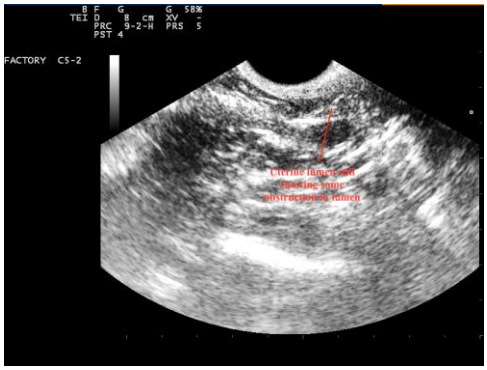
inhibiting the cell proliferation. The results are in accordance with the study done by Youn-Jee Chung and co-workers who demonstrated that Raloxifene when administered with Leuprolide shows more antiproliferative effect on myoma cells. [9]



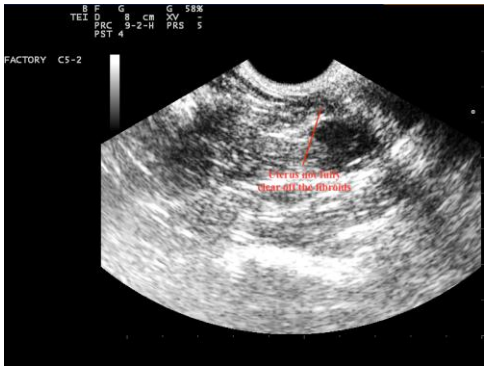
A) LA-15 days



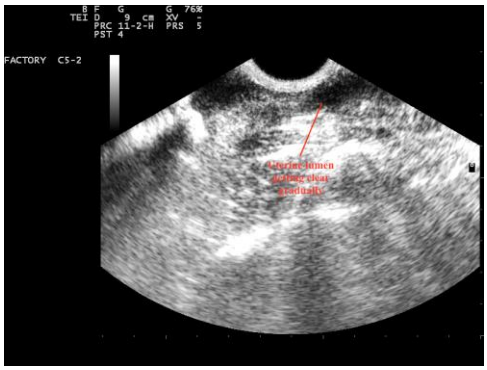
B) LA-30 days



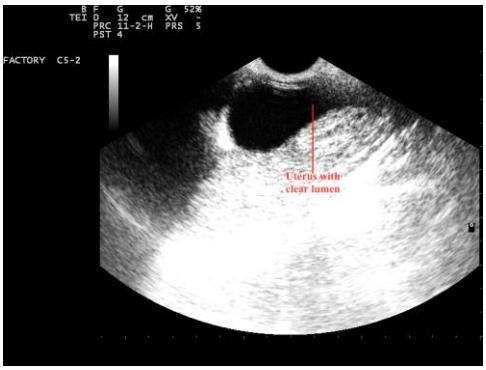
C) RLX- 15 days



D) RLX- 30 days



E) RLX-LA 15 days



F) RLX-LA 30 days

Figure 10.6 Ultrasonography of Rabbit administered with Leuprolide liposomal formulation after A) 15 days B) 30 days; Rabbit administered with Raloxifene liposomal formulation after C) 15 days D) 30 days; Rabbit administered with dual drug loaded liposomal formulation after E) 15 days E) 30 days

Hence, based upon this study we conclude that the effectiveness in fibroid regression is improved by simultaneously administering GnRH analogue i.e. Leuprolide with Raloxifene. The results are in consistent with the conclusions of in vitro cell cytotoxicity study. This research will play a meaningful role in elucidating the mechanisms of medical treatment of uterine myoma.

10.5.2 Results of Pharmacokinetic study

The results of the blood kinetic studies are reported in Table 10.1 showing the concentration of LA and RLX in plasma at various time intervals.

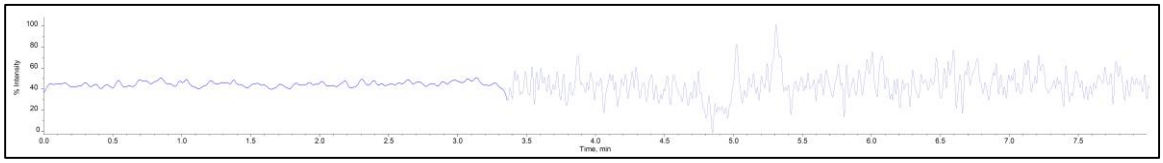
Table 10.1 Administered dose of drug (mg) in rabbit serum after intravaginal administration of formulations

Time (Hours)	**Drug concentration in plasma**	
	Group III rabbit (LA)	**Group IV rabbit (RLX)**
0.25	0	0.027 mg
0.5	0	0.042 mg
1	0.00015 mg	0.061 mg
2	0.00040 mg	0.066 mg
4	0.00077 mg	0.076 mg
18	0.00021 mg	0.024 mg
24	0.000061 mg	0.031 mg

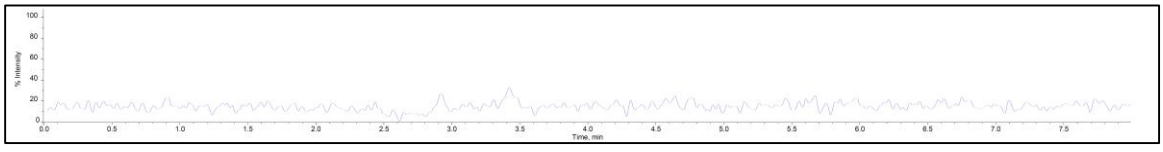
As seen in the results, for group III rabbit administered with liposomal Leuprolide loaded intravaginal rod, first 30 minutes showed no drug in plasma. After 1-hour negligible concentration i.e. 0.05 % of drug administered was quantified in plasma by LCMS-MS.

While in the case of group IV rabbit that was administered with liposomal Raloxifene loaded intravaginal rod, around 1% of the administered dose was estimated in plasma, which accounts for insignificant amount. The presence of drug in plasma could be due to the free drug from the liposomal formulation being uptaken by the plexus of uterus in to the systemic circulation. While the drug entrapped within liposomes was able to accumulate in uterus through the first uterine pass effect phenomena. After 24 hours, the drug concentration in plasma was found to be very less; group III showed only 0.02 % of dose in plasma while group IV had 0.53 %. Very negligible amount of drugs present in the blood will avoid the systemic side effects associated with the drugs. Also the dose needed for the therapeutic effect can be anticipated to be reduced since the drug targeting to the site of action i.e. uterus can overcome the distribution of drugs to other organs. The results of pharmacokinetic studies could be very well correlated with the in vivo biodistribution study showing maximum amount of the drug retained in uterus even after 24 hours. This study also explains the tumor regression occurring in fibroid induced rabbits in the Pharmacodynamic studies. Because the drugs are targeted to the site of action, they act locally on the GnRH and estrogen receptors over expressed on the myoma cells and cause the apoptosis of the cells as seen in the in vitro cell culture studies.

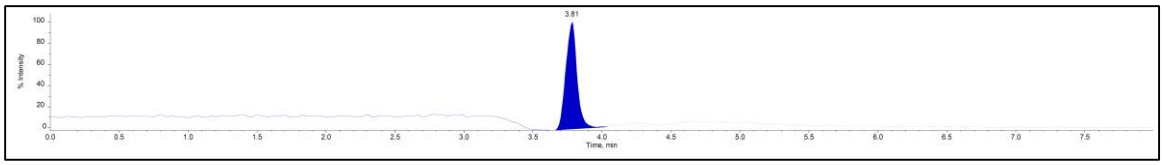
Figure 10.7 and 10.8 shows the chromatograms of LA and RLX, respectively, in rabbit plasma at various time intervals. Table 10.2 gives the peak areas of LCMS chromatograms.



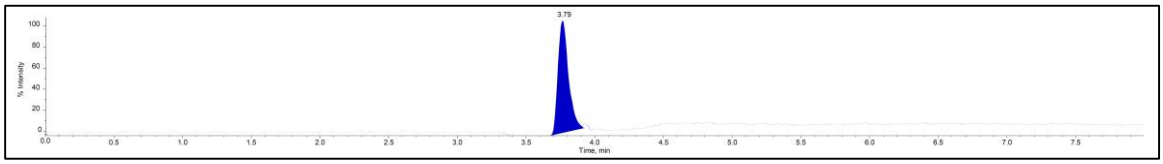
0.25 hour



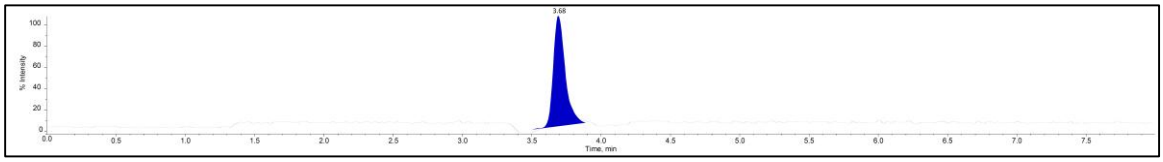
0.5 hour



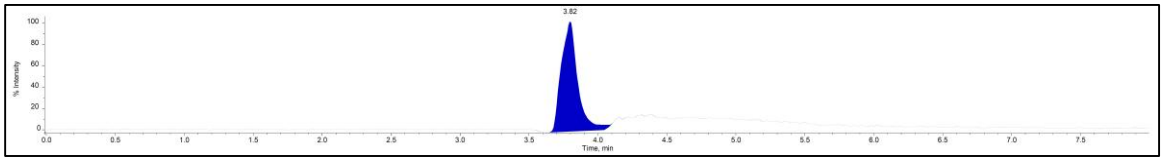
1 hour



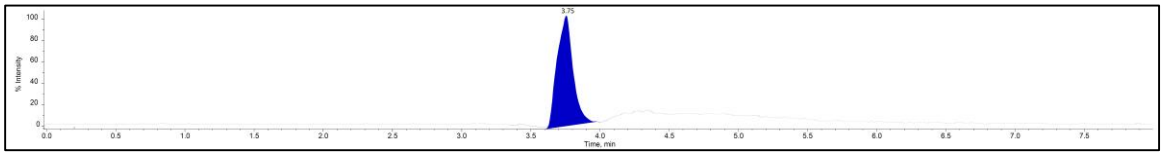
2 hours



4 hours

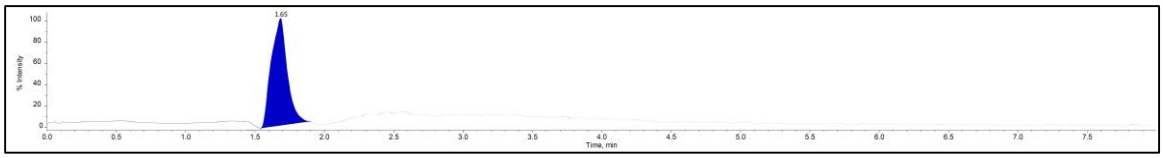


18 hours

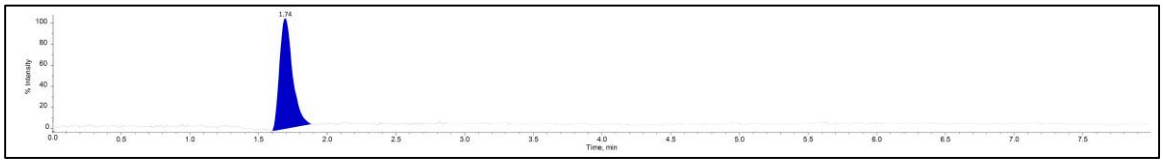


24 hours

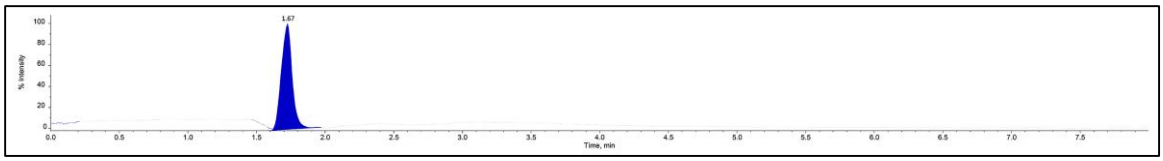
Figure 10. 7 Chromatograms of LA in rabbit plasma at various time intervals



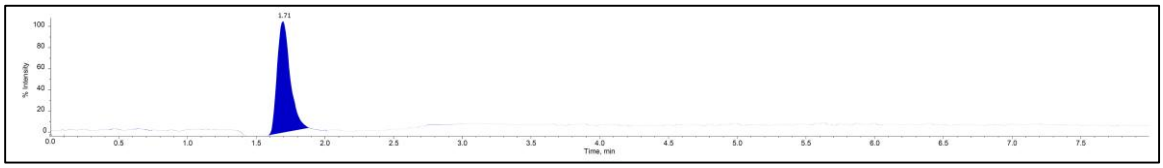
0.25 hour



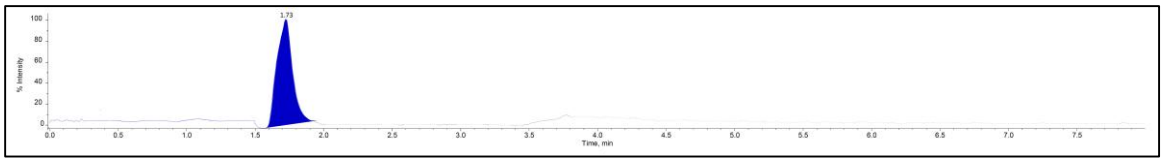
0.5 hour



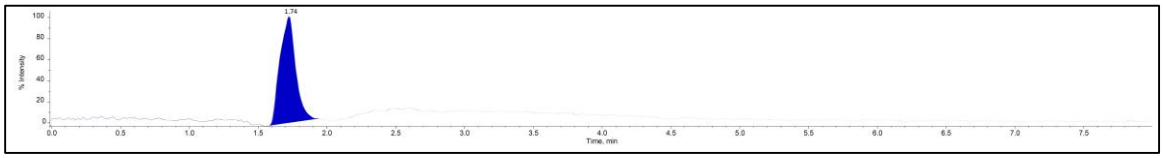
1 hour



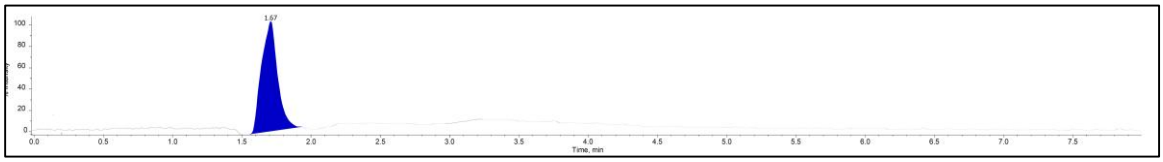
2 hours



4 hours



18 hours



24 hours

Figure 10.8 Chromatograms of RLX in rabbit plasma at various time intervals

Table 10.2 Peak Areas of LCMS Chromatograms

Time (Hours)	Peak Areas	
	LA	RLX
0.25	0	442001319
0.5	0	687551320
1	17885297	1002000000
2	46487297	1080000000
4	89390297	1247000000
18	25035797	402713319
24	7159547	520577319

10.6 References

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