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Full Length Research Paper

Preclinical 28 days sub-chronic toxicity study of novel formulation of metoprolol tartrate on albino rats using statistical tool

Sabahuddin Siddique¹, Sourav Das², Mohi Iqbal Mohammed Abdul^{3*}, Syed Ata Ur Rahman³, Durdana Lateef⁴, Shubhasis Dan² and Anirbandeep Bose²

¹Patel College of Pharmacy, Department of Pharmacology, Madhyanchal Professional University, Bhopal, M.P., India.

²TAAB Biostudy Services, Jadavpur, Kolkata, India.

³College of Pharmacy, Department of Pharmacology, Taibah University, Al-Madinah Al-Munawwarah, Kingdom of Saudi Arabia.

⁴College of Science, Department of Mathematics, Taibah University, Al-Madinah Al-Munawwarah, Kingdom of Saudi Arabia.

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The experimental study mainly focused on evaluation of sub-chronic oral toxicity of sustained formulation of metoprolol succinate. After the formulation development of multiparticulate sustained release capsules, there was an urgent need to analyze their *in vivo* bioavailability in healthy normal human volunteers. Prior to use in human population, a subchronic oral toxicity study was performed on Swiss Albino rats, of the final formulated product to assess the possible therapeutic outcome and related toxicity. The final objective of the study is to find out the pharmacological interaction between the drugs and the excipients that may lead to toxicity to human volunteers. The sustained release formulation of Metoprolol tartrate was administered in different dose according to the 28 days of study dosing schedule in 48 albino Wistar rats of either sex (24 males and 24 females); periodical safety and efficacy observation were done followed by blood chemistry, hematology and histopathological examination at the end of the study. All the animals were found alive after the study period. No behavioral abnormalities were found in the study animals. Statistical analysis of the results including various parameters like body weight, blood chemistry, hematology and histopathological evaluation did not produce any major differences between control and treated groups. The reformulated sustained release hydroxy propyl methyl cellulose based granules of metoprolol tartrate did not cause any subchronic toxicity to the study animals under experimental conditions.

Key words: Preclinical sub-chronic toxicity study, rats, formulation, metoprolol tartrate.

INTRODUCTION

The urge for discovery of novel drugs has generally emerged for the treatment of incurable life threatening

diseases and as well as for better management and treatment of mild to moderate diseases. After the

*Corresponding author. E-mail: iqbalmohi100@gmail.com.

discovery of the drugs it must pass through a series of clinical study. After thorough review of the clinical study the regulatory body will ultimately allow for next stage of marketing of the drug. Although raw drugs are safe as they are undergone various tests and check up but when the drug is formulated in various dosage forms it may show some sort of toxicity. Due to use of various types of ingredient in pharmaceutical dosage form they may interact with drugs. As a result the drug may show some toxicity effects. These drugs must fulfill stringent requirements to become successful in clinical therapy (Semih and Ahmet, 2014).

The ability of a drug to meet these requirements is dependent not only on the physicochemical properties of the drug itself but also on the design of the dosage form in which it would be dispensed. The success of the dosage form is also dependent on the extent it can combat with patients' psychology. The duty of a pharmaceutical scientist is to figure out the best environment for the drug in which it would be most effective in terms of clinical and aesthetic efficacy. In this way pharmaceutical product development is a science as well as an art. Research in this area is progressing with new drug, and novel drug delivery systems (NDDS) are always emerging to deliver them in a better way than before. The number of products based on NDDS has significantly increased recently. They can improve therapy by increasing the efficacy and duration of drug activity (Shubashish Dan et al., 2016).

Some act by increasing patients' compliance through decreased dosing frequency and convenient routes of administration. Others are reported to improve targeting for a specific site to reduce unwanted side effects. Still, a number of them mimic the circadian rhythm of particular diseases in order to optimize a drug's therapeutic power, potentially differentiating a brand and giving it a competitive edge over less effective drug (Kaushal and Garg, 2003).

Oral drug delivery system is the most popular route, which is due in part to the ease of administration and to the fact that gastrointestinal physiology offers more flexibility in dosage form design than most other routes (Gupta et al., 1992). There is a plethora of oral controlled release products in the market place. In 1998, the U.S Food and Drug Administration (FDA) approved 90 oral controlled release products; from 1998 to 2003, FDA approved an additional 29 new drug applications that used controlled release technologies and 12 of them were based on matrix systems (Hu et al., 2006).

Development of oral controlled release dosage forms of a given drug involves optimization of the dosage form characteristics within the inherited constraints of the gastrointestinal physiology. Controlled release delivery systems have added advantages over immediate release dosage form. Since the frequency of drug administration is reduced, patients' compliance can be improved and

drug administration can be more convenient (Hayashi et al., 2005; Nokhodchi and Tailor, 2004).

It also causes less fluctuation of plasma drug level and leads to more uniform drug effect and lesser total dose. On the other hand, controlled release dosage forms have some disadvantages which include generally higher cost, relatively poor in vitro/in vivo correlation, unpredictable and even reduced bioavailability and subjected to increased first pass metabolism for certain drugs. In order to exert control over the rate of the drug release, as well as movement of the dosage form through the gastrointestinal tract, a number of factors such as motility, pH, ionic strength of luminal content and differential absorption must be considered (Gupta et al., 1992).

Development of oral sustained release formulations for highly water soluble drugs with constant rate of release has become a challenge to the pharmaceutical technologists. In general, fast release of drug causes toxicity if it is not formulated as extended release dosage form. Metoprolol tartrate (MT), a β - blocker being highly soluble, according to BCS classification permeable (class I substance) is absorbed completely through the whole intestinal track within 2-4 h. It is subjected to extensive first pass metabolism. Its low biological availability (~50%), quick absorption and elimination (3-4 h) necessitate the administering of conventional IR up to 4 times daily (Al-Saidan et al., 2004; Siddique et al., 2011). To overcome this frequent dosing problem sustained release is developed that enables less frequent dosing. In the present study, matrix granules of MT have been formed by suitable combination of hydroxypropyl methyl cellulose (HPMC) and ethyl cellulose (EC). Eudragit® RL and RS were chosen to form coating on the granules to extend duration of drug release.

The new formulation 'Diffusion Controlled Coated Matrix System (DCCMS)' or (C1) has undergone instrumental analysis (XRD, DSC and FTIR) to ensure compatibility between drug and associated polymers, excipients. Commonly used instrumental analysis may not reveal the presence of trace amount of toxic by-product generated during synthesis of drug and McDonald, 1995). Oral administration of metoprolol tartrate is safe and well established, but as different polymers and other excipients have been used to develop the formulation, there may be a possibility of incompatibility and enhancement of toxicity. A cardiac patient generally uses an antihypertensive drug for years till the remaining part of his/her life. Toxicity may develop on continuous use of the drug. So it is mandatory to perform 'Subchronic Oral Toxicity Study' of newly developed formulation (Madhusudhan et al., 2017).

The joint expert committee on 'Food Additives' had set typical criteria for the evaluation of safety; such as physical appearance and behavior, growth and body weight gain, food consumption (Food additives, 2000). Evaluation of feed utilization, plasma hematology and

blood chemistry, gross pathological examination on necroscopy, organ weight and histopathology are also recommended. LD50 in male rats after oral administration of MT is 90 mg/kg (Neil et al., 2001).

A rigorous safety evaluation of this new formulation would assist in establishing it as sustained release formulation for highly water soluble drug metoprolol tartrate. Therefore a short time sub chronic toxicity study was conducted in rats by feeding freshly prepared suspension that was made out of sustained release granules of metoprolol tartrate. To evaluate the evidence of toxicity 28 days short term subchronic toxicity study was executed according to OECD guidelines (OECD, 2008).

The aim of our experiential study is to access toxicity profile of the drug in a systemic manner for 28 days. The experimental toxicity study of these drugs will generate the idea of whether the interaction of polymer with active pharmaceutical ingredient produces any toxic impurities that may cause serious toxicity in long term and short term uses.

MATERIALS AND METHODS

Materials

Metoprolol tartrate (98 to 101% purity) was received as gift sample from Torrent Laboratories, India. Other excipients used in formulation like hydroxy propyl methyl cellulose (HPMC K100M), Ethyl Cellulose, (EC) (Ethocel*FP Premium, 7 cps viscosity grade), HPMC E5 and dicalcium phosphate (DCP), Eudragit® RS and RL were received as donated samples by Dhara Life Science Pvt. Ltd. India. All the pharmaceuticals belong to Pharmacopoeial standard (USP/NF). Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from M/s. Qualigens Fine Chemicals, Mumbai, India.

Experimental design and conduct

Randomization, numbering and grouping of animals

Forty eight albino Wistar rats (aged 7 to 8 weeks; 24 male and 24 female healthy rats) were divided into four groups of 6 rats per sex: four dose groups received 0, 15, 30 and 60 mg/kg doses. All the rodents were allowed to acclimatize for 7 days to laboratory conditions prior to the initiation of dosing. Each cage contained 6 rats of the same sex with a bedding of husk, and 12 h light/dark cycles were provided (Hirst et al., 2014). The individual animal was fur marked with picric acid. The females were nulliparous and not pregnant. Pelleted-feed and water were given *ad libitum*. Animals were kept in animal house maintained at a temperature of 22°C ± 2°C and a relative humidity of 55 ± 5%. The study protocol was approved by the Institutional Animal Ethical Committee. Table 2 displays the allocation of animals into different groups.

Preparation of test material

The test formulation (C1) was crushed to powder by mortar and pestle and dispensed into suspension using 100 ml purified water

so that the concentration of the pure drug in the suspension was 1 mg/ml. The HPMC polymers in the formulation acted as suspending agent. On each day of the study freshly prepared suspension was administered to the rats between 9-10 a.m. for 28 days. The drug was then administered to rats at the dose levels of 15, 30 and 60 mg/kg in the dose volume of 1ml/ 100g body weight. The control animals were fed with vehicle only.

Observations

All animals were observed daily for clinical signs. The time of onset, intensity and duration of these symptoms, if any, were recorded. All animals were observed twice daily for mortality during the period of the study. The weight of each rat was recorded on first day at 0 h and at weekly intervals throughout the course of the study. 'Mean body weight' of each group was calculated. The quantity of food consumed by groups consisting of six rats each was recorded weekly and the food consumption per rat was calculated for both the 'control group' and 'groups fed with dose'. Tables 3 and 4 display the body weight of the animals and their food consumption.

Terminal studies

Laboratory investigations

The following investigations were carried out prior to sacrifice on completion of dosing period of 28 days in the animals that were fasted over-night. Blood samples were collected from orbital sinus the next morning using heparin as anticoagulant (Parasuraman et al., 2010).

Haematological investigations

The haematological parameters hemoglobin concentration (Hb), mean corpuscular volume (MCV), total erythrocyte count (RBC), reticulocyte concentration (Rt), mean corpuscular hemoglobin (MCH), hematocrit (HCT), and total and differential leucocyte count were determined using hematology analyzer (RmdMediaids Pvt. Ltd., New Delhi, India). Serum was obtained by centrifuging at 3000 rpm for 10 min and supernatants were taken to determine serum glucose, total protein, albumin and blood urea nitrogen, sodium, and potassium analyzed using autoanalyzer (RmdMediaids Pvt. Ltd., New Delhi, India). Hematological parameters: hemoglobin (gm %) (Hb), reticulocyte (%) (Rt), platelets ($\times 10^5$ /cmm), white blood corpuscles ($\times 10^3$ /mm³) (WBC) were studied using Sysmax – K250 Cell Counter and results are given in Table 5a and 5b.

Biochemical investigations

Biochemical parameters total serum protein (gm %) (Henry et al., 1957) blood urea nitrogen (mg %) (BUN) (Kaplan et al., 2006), serum glutamic pyruvic transaminase (IU/L) (SGPT), and serum glutamic oxaloacetic transaminase (IU/L) (SGOT) were studied (Reitnan and Frankel 1957) using Robonik ASP-300 and results are given in Table 6a and 6b.

Necropsy

All animals were sacrificed on day 29, using CO₂ asphyxiation technique. Necropsy of all animals was carried out and the weights of the following organs were recorded: Liver, kidneys and heart.

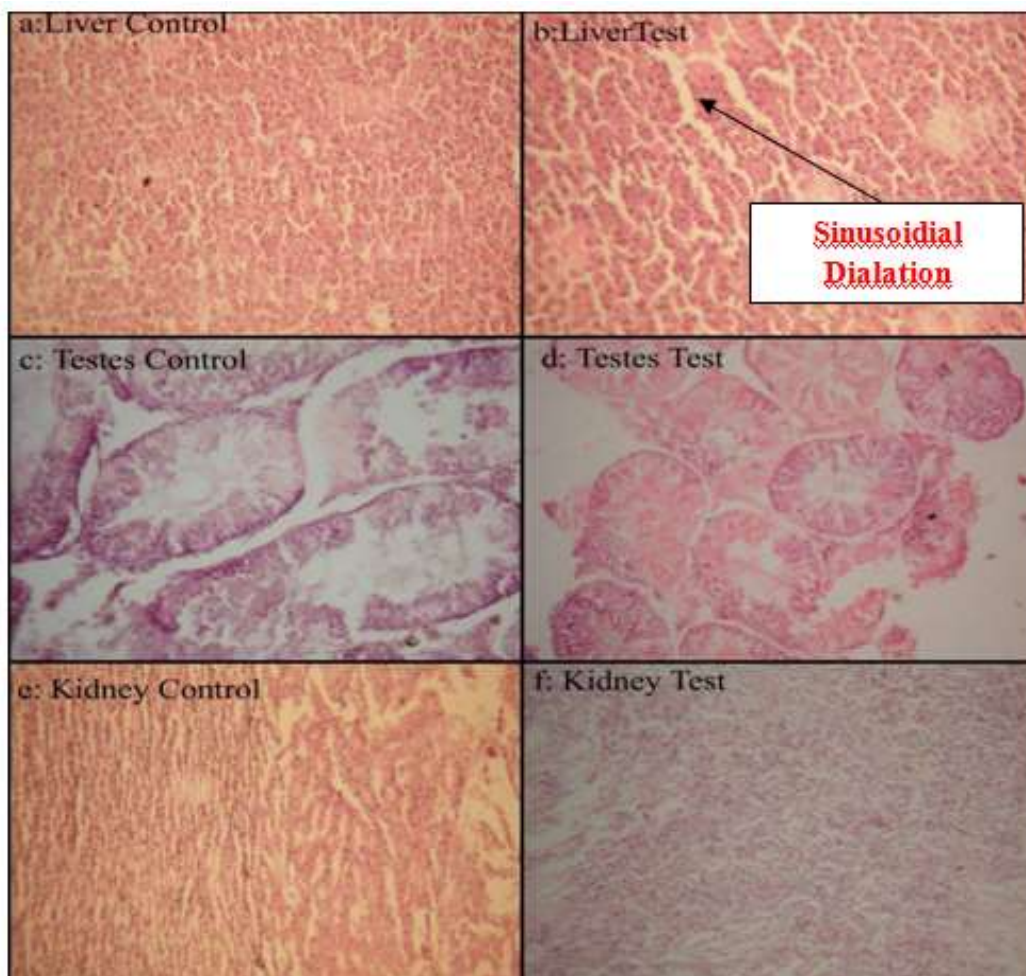


Figure 1. Histopathological examination of the liver, kidney and testes in the control and the test groups.

The organ weights were recorded as absolute values and their relative values (that is per cent of the body weight) were calculated.

Autopsy and histopathology

Body of anaesthetized animal was dissected and all organs were observed macroscopically, selected organs were excised, weights of organs were recorded and % change in body weight was calculated. Then these organs were fixed in 10% buffered neutral formalin solution. Paraffin sections were prepared and stained with hematoxylin and eosin for histopathological examination. All slides were examined by a pathologist. The following tissue samples of organs from control and animals treated with the highest dose level of 60 mg/kg were preserved in 10% formalin for histopathological examination: Adrenals, heart, kidneys, liver, lungs and stomach.

Adrenals, heart, kidneys, liver, lungs and stomach of 'low and intermediate dose group' animals were preserved for possible histopathological examination, in case the histopathological examination of high dose group animals is indicative of abnormalities associated with the treatment. Figure 1 displays histopathological sections of different organs.

Statistical evaluation

Four groups of 12 rats (6 males and 6 females) were assigned into four different treatments: control, low dose, middle dose and high dose. After 28 days all pathological and biochemistry parameters were evaluated statistically. Analysis of variation (ANOVA) enables us to compare the means of three or more variables. The test compares the variation (variance) in the mean between treatments with those within treatments. The ratio of variations (variance) in the mean between treatments with those within treatments determines the F value (Shravan et al., 2011).

Feed conversion efficiency percentage was calculated as follows:

$$\% \text{ Feed conversion efficiency} = \frac{\text{Weekly food consumption (grams)}}{\text{Weekly body weight gain (grams)}} \times 100$$

Histopathological observations were carried out in control and animals treated with the highest dose level of 60 mg/kg. Tissue samples were preserved in 10% neutral buffered formalin (Luciana et al., 2012).

Statistical analysis was done using analysis of variance

Table 1. Allocation of animals to various groups.

Group number	Dose (mg/kg)	Sex	Number of animals in each group	Animal numbers
I	Control	Male	6	1-6
		Female	6	7-12
II	15	Male	6	13-18
		Female	6	19-24
III	30	Male	6	25-30
		Female	6	31-36
IV	60	Male	6	37-42
		Female	6	43-48

Table 2. Group mean body weight (g) of animals.

Group number	Dose (mg/kg)	Day				
		0	7	14	21	28
I	Control	110.46±6.75	112.63±4.78	118.78±7.23	119.34±6.40	120.28±5.82
II	15	112.29±6.45	113.14±5.68	117.56±7.12	120.52±4.57	122.12±5.43
III	30	108.86±6.34	109.67±7.45	113.42±6.56	118.31±5.12	119.18±4.51
IV	60	107.69±6.62	108.27±5.34	119.31±5.81	115.08±4.75	123.32±5.21

p < 0.05.

(ANOVA). Student t test was employed to compare the statistical significance between control and experimental groups (Aboudoulatif et al., 2010; Bidhe eand Ghosh, 2004).

RESULTS

Sub chronic oral toxicity study (28 days) on rats

Sub chronic oral toxicity study for reformulated drug before *in vivo* study was accomplished. 48 rats weighing 100 to 150 g were treated in 4 different doses including control group shown in Table 1.

Clinical signs of rats

All animals (48) were free of intoxicating signs throughout the dosing period of 28 days.

Mortality of animals

Male and female animals from control and different dose groups survived through the dosing period 1 of 28 days,

indicating no mortality and drug safety and no significant behavioral abnormality were observed.

Variation of body weight of experimental rats

Both male and female animals from control and the different dose groups exhibited normal body weight gain throughout the entire study duration of 28 days as illustrated in Table 2 (body weights mentioned in the section are fasting body weights).

Food consumption of experimental animals (male and female)

During the dosing period and at termination the quantity of food consumed by both male and female animals from different dose groups was found to be comparable with that of control animals.

Hematological investigations of blood parameters

The hematological parameters of both male and female animals at termination of dosing on day 29th had no

Table 3. Group mean food consumption (g/ animal)

Group number	Dose (mg/kg)	Mean	D a y				
			0	7	14	21	28
I	Control	Mean	15.3±1.9	16.1±1.8	16.4±1.7	17.0±1.8	17.8±1.6
II	15	Mean	14.2±1.4	15.1±1.5	16.0±1.5	16.3±2.0	16.9±1.5
III	30	Mean	14.7±2.1	15.0±1.4	15.3±1.3	16.0±2.2	16.5±1.3
IV	60	Mean	13.7±1.9	14.0±1.3	14.5±1.2	15.9±1.9	16.2±1.1

p <0.05.

Table 4a. Group mean – hematology of animals.

Group number	Dose (mg/kg)	Hb (g %)	Platelets ($10^5/\text{mm}^3$)	Rt (%)	Total WBC ($\times 10^3/\text{mm}^3$)
I	Control	16.17±0.88	7.3±0.38	1.6±0.16	7.06±0.48
II	15	16.02±0.49	7.45±0.32	1.51±0.1	7.08±0.20
III	30	16.39±0.71	7.55±0.37	1.58±0.18	6.98±0.44
IV	60	15.94±0.36	7.44±0.43	1.18±0.15	7.20±0.68

Table 4b. ANOVA of Hematological parameters of 4 different groups (No. of subjects, 48) under subchronic toxicity studies.

Name of the parameter	Mean square (between treatment)	Mean square (within treatment)	F- Value	Statistical significance
Hemoglobin	0.468	0.412	1.138	NS
Platelets ($\times 10^5/\text{mm}^3$)	0.118	0.140	0.838	NS
Rt (%)	0.026	0.023	1.132	NS
Total WBC ($\times 10^3/\text{mm}^3$)	0.10	0.23	0.41	NS

Table 5a. Group mean – blood chemistry.

Group number	Dose (mg/kg)	Total serum protein (g %)	BUN (mg %)	SGPT (IU/L)	SGOT (IU/L)
I	Control	7.14±0.48	18.58±2.61	18.08±1.16	24.19±1.0
II	15	6.98±0.30	17.67±1.61	17.33±1.15	24.72±0.85
III	30	7.07±0.44	17.41±1.08	17.67±1.23	25.06±1.06
IV	60	7.2±0.68	17.5±1.0	17.83±1.59	22.76±1.0

Table 5b. ANOVA of biochemical parameters of 4 different groups (No. of animals,48) under sub chronic toxicity studies.

Name of the parameter	Mean square(between treatment)	Mean square(within Treatment)	F- Value	Statistical significance
Total serum protein	0.10	183.191	0.005	NS
BUN (mg %)	3.47	2.89	1.198	NS
SGPT	1.187	1.679	0.707	NS
SGOT	12.322	13.777	0.894	NS

Table 6a. Group mean – relative values (%) of different organ weights in different groups.

Group number	Dose mg/kg	Liver (g)	Kidneys (g)	Heart (g)
I	Control	4.69±0.32	0.85±0.04	0.58±0.03
II	15	4.78±0.25	0.84±0.06	0.60±0.04
III	30	4.86±0.24	0.87±0.03	0.61±0.04
IV	60	4.95±0.24	0.87±0.03	0.59±0.03

Table 6b. ANOVA of histological parameters of 4 different groups.

Name of the parameter	Number of subjects	Mean square(between Treatment)	Mean square(within Treatment)	F -Value	Statistical significance
Liver	48	0.142	0.070	2.031	NS
kidney	48	0.002	0.001	1.318	NS
Heart	48	0.002	0.001	1.715	NS

significant changes in the values of different parameters studied (Table 4a and 4b) when compared with controls; values obtained were within normal biological and laboratory limits. The ANOVA was applied to evaluate the statistical difference of the mean hematological parameters among four independent drug treatments. Since the value for F obtained in the present experiment is less than the recorded value, it was concluded that the difference between the treatment means is not significant ($p < 0.05$).

Biochemical Investigations of male and female animals under sub-chronic toxicity study

Male and female

At termination on day 29, all biochemical parameters studied, total serum protein; SGPT, SGOT, BUN were found to be comparable with controls and were within the normal biological and laboratory limits. The ANOVA was applied to evaluate the statistical difference of the mean biochemical parameters among four independent drug treatments. Since the value for F obtained in the present experiment for different biochemical parameters was lesser than the recorded value, the difference between the treatment means was not significant ($p < 0.05$).

Variation of organ weights of male and female animals under sub-chronic toxicity study

Male and female

The animals from control and the different dose groups exhibited normal organ weight after the sacrifice at 29th

day. The ANOVA was applied to evaluate the statistical difference of the mean weight of vital organs among four independent drug treatments. Since the value for F obtained in the present experiment for different organ weights was less than the recorded value, we conclude that the difference between the treatment means was not significant ($p < 0.05$).

Necropsy

The gross pathological examination revealed no abnormality attributable to the treatment.

Histopathology of different organs of high dose groups

Summary of histopathological observations of vital organs of different dose groups is given in Figure 1. Histopathological examination of animals from high dose group revealed no abnormality attributable to the treatment. Histopathology findings of different vital organs (kidneys, liver, testis) of control and high dose (60 mg/kg) animals revealed severity level of 6 which was normal.

The histopathological changes are shown in Figure 1. Some morphological change was observed in transverse section of liver after the use of our test drug at highest concentration level. Sinusoidal Dilatation was found in liver transverse section of animal receiving test drug. Sinusoidal dilation in liver cell can be caused with a small impairment in liver function (Siddique et al., 2011). Though, it was also described in some literatures (Laffón et al., 1989; Dan et al., 2016) that the manifestation of the hepatotoxicity could also be because of some pathogenic attacks, which may lead to some genomic alteration. The

liver is capable of regenerating damaged tissue, so, if the study drug is found to be responsible for the manifestation of liver function, it would not harm the patient after withdrawal of toxicant " previously it was observed in table 5a, that the level of liver enzymes was in normal range, the hepatic impairment in Albino rats is not a huge concern". The other morphology study of kidney and testis did not show any drastic histopathological changes.

DISCUSSION

The design of dosage form was performed by choosing hydrophilic (hydroxypropyl methyl cellulose) and hydrophobic (ethyl cellulose) polymers as matrix builders and Eudragit® RL/RS as coating polymers. Granules were prepared by composing drug, hydroxypropyl methyl cellulose (HPMC K100M), ethyl cellulose (Ethocel*FP Premium, 7 cps viscosity grade) (EC), dicalcium phosphate by wet granulation method followed by coating. Formulation CMG25 formed by using 30% HPMC K100M, 20% EC, and ratio of Eudragit® RS/RL as 97.5:2.5 at 25% coating level gave best micromeritic and *in vitro* results. Capsules were filled by free flowing granules of uniform drug content. This extended the release period up to 12 h *in vitro* study. Formulation CMG25 was further optimized to get the desired release of metoprolol tartrate by RSM technology. The optimized formulation was coded as C1 (Tomisla et al., 2012).

It was observed that the animals fed with the formulation (C1) suspension were healthy. No unusual changes in behavior or in locomotor activity, no ataxia, and no signs of intoxication were observed during the 28-day period. No differences were found in growth between the control group and the test animals. The food consumption of male and female rats of control and experimental groups was similar, indicating that the feed intake and utilization was not affected (Gopi et al., 2016).

Observations of gross pathology immediately after dissection on rats of all groups were found to be uniformly healthy, showing no apparent pathological abnormalities. Histopathological examination of the liver and kidneys in the control and the test groups was carried out; a slight morphological alteration was monitored, but it needs more evidence to conclude that the change is due to the study formulation. Otherwise, the study revealed that formulation did not cause any adverse toxicological effect on organs. Haematological parameters, hemoglobin concentration, total and differential erythrocyte count, total and differential leucocyte count, hematocrit, and mean cell hemoglobin concentration, in both control and experimental rats, indicated no significant difference ($P < 0.05$) between the two groups. All values were found to be within the normal range for rats (Brij and Ogunkunle, 1981) and there were

no differences between the groups.

The levels of plasma analytes, such as total protein, blood urea nitrogen (BUN), glucose, bilirubin, albumin, creatine, cholesterol, chloride, calcium, phosphorus, sodium and potassium ions were not significantly different between the control and the experimental groups of rats ($P < 0.05$). No significant differences were observed in enzyme activities between the control and test animals. No abnormal changes were observed in organ mass with respect to body mass of the test rats in comparison with control.

Conclusion

After development of formulation, there was a need to evaluate their *in vivo* performance. So prior to this, a sub chronic oral toxicity study of the formulation was carried out in rats to determine any toxic effect due to interaction between the drugs and the excipients. The toxicity study of any new formulation is important to ensure safe administration of the formulation to human volunteers for biopharmaceutical evaluation (Lateef et al., 2016). The purpose of this study is to look at the toxicity profile of the new sustained release formulation of metoprolol tartrate using minimum number of animals. In this sub chronic study of 28-day, no significant differences were observed in the body weight, hematological parameters, plasma analytes, enzyme activities and histopathological findings between the control and test animals, suggesting that the formulation may not be toxic. In conclusion, the test material did not cause any adverse effect on the albino rats under experimental conditions. So its oral administration to human volunteers may be safe for further preclinical study. In future bioavailability study can be carried out in human volunteers.

CONFLICT OF INTERESTS

The author has no conflict of interest.

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