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Original Research Article

An *In-Vivo* Evaluation of Chronotherapeutical Drug Delivery System for the Treatment of Hypertension

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Abstract: Chronotherapeutics refers to a treatment method in which in vivo drug availability is timed to match rhythms of disease, in order to optimise therapeutic outcomes and minimise side effects. The term "chrono" basically refers to the observation that every metabolic event undergoes rhythmic changes in time. It refers to a treatment method in which *in vivo* drug availability is timed to match rhythms of disease in order to optimize therapeutic outcomes and minimize side effects. It is based on the observation that there is an interdependent relationship between the peak-to-trough rhythmic activity in disease symptoms and risk factors, pharmacologic sensitivity, and pharmacokinetics of many drugs. In this present study *invivo* evaluation of bilayered tablets of Losartan and Hydrochlorothiazide were studied on rabbits to prove that the prepared formulation shows a pulsatile drug release. The plasma levels were measured using LC-MS/MS method. Pharmacokinetic parameters of these tablets were determined by using WINNOLIN Scientific software. The pulsatile release tablet formulation prepared in the lab managed to show some lag phase initially before releasing the drug with a maximum time (Tmax) at the 12th hour. From this present work the results of invivo studies proved that chronotherapeutical release of drug in the form of bi layered tablet can be achieved for newer chronotherapeutical drug delivery system.

Keywords: Chronotherapeutics, *In-vivo*, Pharmacokinetic Parameters, WINNOLIN

INTRODUCTION

Recent advances in chronotherapeutics led to the development of pulsatile drug delivery systems which effectively delivered the drug at specified time. Diseases like asthma, arthritis, cancer, diabetes, hypertension, ulcer, hypercholesterolemia, congestive heart failure, stroke etc. show different day night pattern in onset and symptoms exacerbation. Pulsatile drug delivery systems deliver the drug at right time in desired levels providing the multiple benefits over the conventional dosage forms. According to the circadian rhythms of the body drug is facilitated to completely release after a lag time especially for drugs eliciting higher first pass effect and where nocturnal dosing is required these systems are highly beneficial. This review epitomizes the special focus on chronotherapeutics, various approaches in chronotherapeutic drug delivery and applications. Master circadian clock of the body, the suprachiasmatic nucleus regulates the endogenous circadian rhythms present inside the human body Major global market of drug delivery systems is occupied by the oral drug delivery systems where the drug release pattern is within the therapeutic window assures the sustained therapeutic action .some conditions demands release of drug after a lag time, i.e., a period of no drug release, where pulsatile drug delivery releases the drug completely after a lag time with increased patient compliance shown in Figure 1**.** Lag time is essential for site specific drug delivery to colon requiring the prevention of drug in G.I.T excessive first pass metabolism, drug degrade in gastric acid medium in stomach, which results in bioavailability. Human body functions such as metabolism, behavior sleep patterns, hormone production regulated by circadian rhythms. Reports suggests that more chances of heart attacks in the early morning hours ,high levels of cortisol levels ,blood pressure were also high early morning than drops off in the night Nocturnal asthma increased responsiveness in early hours of morning, sudden surge of gastric acidity in the mid night. High cholesterol synthesis in night than in the day light all these events associated with the circadian rhythms definitely reveals the importance for designing time specific drug delivery [3]. In evaluating a chronotherapeutical drug delivery system, a fundamental issue is the types of studies that should be performed to give reasonable assurance of safety and efficacy. While providing important information concerning the release characteristics of the drug from the dosage form, at present in vitro studies

are most important and useful for such purposes as monitoring the drug product stability and manufacturing process control. The assessment of safety and efficacy of a pulsatile dosage form is best achieved through observing the in vivo pharmacodynamics and pharmacokinetics. Moreover, where there is a well defined, predictive relationship between the plasma concentration of the drug or active metabolite and the clinical response , it may be possible to use drug plasma drug concentration data alone as a basis for the approval of the dosage form that is designed to replace an immediate release preparation.

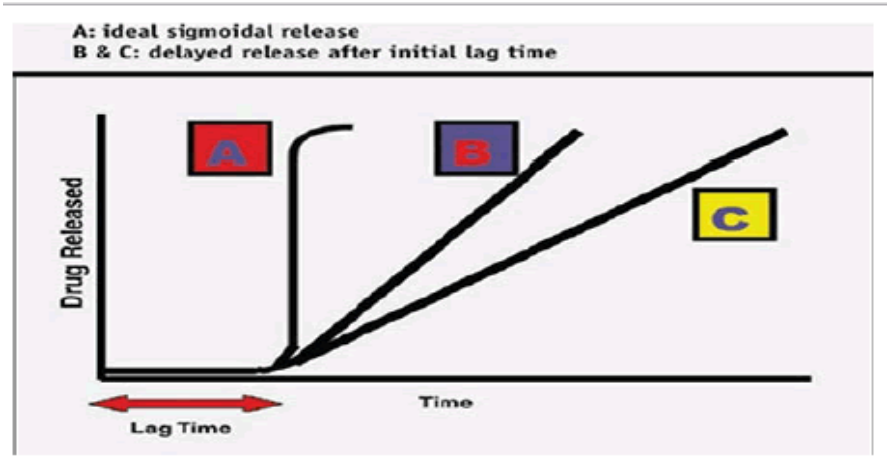

Fig-1: Drug release profile of pulsatile drug delivery systems

Hence the objective of the current investigational work is to *in-vivo* evaluate the chronotherapeutical drug delivery system of Losartan and Hydrochlorothiazide in the form of bi layered tablets for the treatment of hypertension.

MATERIAL AND METHODS

Losartan Hydrochloride and Hydrochlorothiazide was obtained as gift sample from Sanofi India Limited, Goa. Rest of the Polymers were purchased from different suppliers from Bangalore.

In Vivo Evaluation Studies [4-7]

The studies in rabbits were conducted after obtaining approval from the Institutional Animal Ethics Committee of the K.S. Hegde Medical Academy, Deralakatte, and Mangalore. New Zealand white rabbits of either sex and body weight of 2.5-3.0 Kg were used for the test. To carry out the study, the formulation was orally administered to the rabbits. Before the test, rabbits were fasted overnight with ad libitum, having stored them in individual cages for acclimatization period of two weeks before the experiment was carried out. The rabbits were divided into groups of 2 each as Group A and Group B. The study was conducted in a crossover design with 3 weeks washout periods in between the two experiments. The tablet was administered using sterile internal stomach pumps.

Table 1: Formulations administered to different groups of rabbits for *in vivo* studies

Group	Formulation
Group - A	Oral solution of Losartan (2.0 to 4.0 mg)
Group - B	Pulsatile tablet of Losartan

Procedure for Blood sampling

Using clippers, hairs were removed from the ear. Ear was cleaned with 95% v/v alcohol and local anaesthetic cream (EMLAP) was applied on the collection site 10 min prior to sampling. Blood samples (5 ml) were collected from the tracheal lobular vein of the rabbit using 22 guage needle and the blood was stored in screw top heparinized plastic tubes, the sampling time for blood was done at 0 mins (predose), 15 mins, 30 mins, 60 mins, 120 mins, 180 mins, 240 mins, 480 mins, 600 mins, 720 mins,1200 mins and 1440 mins The plasma was immediately separated by aspiration after centrifugation at 4000 rpm for 5 minutes and frozen at -20 ºC until analyzed.

Preparation of the Internal Standard (IS) stock solution.

About 2mg of internal standard (Losartan) was weighed accurately & transferred into a 2ml volumetric flask. It was then dissolved in Methanol and the volume was made up with the same to produce a solution of 1mg/ml strength of internal standard. The above final concentration internal standard was corrected according

to its potency and actual amount weighed. It was then stored in refrigerator or cooling cabinet.

Preparation of Losartan standard stock solution.

About 2mg of Losartan working standard was weighed accurately and transferred into 2ml of volumetric flask. It was then dissolved in methanol and the volume was made with the same to produce a solution of 1mg/ml strength of Losartan. The above final concentration for losartan was corrected for accounting for its potency and the actual amount weighed. It was then stored in refrigerator or cooling cabinet.

Spiking of plasma for Samples.

0.7ml of each of the described stock dilution of Losartan was transferred into a 10ml of volumetric flask and the volume was made up with Sodium heparin .Plasma then was pooled and mixed well.

Procedure for Sample Preparation.

All samples of one or more periods were withdrawn from the freezer or deep freezer and allowed them to thaw at room temperature. The thawed samples were vortexed to ensure complete mixing of contents. 100µl of samples were pippeted into respectively labelled Radio-Immuno Assay (RIA) Vials. 50µl of internal standard (0.5µg/ml) were added into respectively labelled RIA vials and vortex. 0.5ml of extraction solvent (Ethyl Acetate) were added to all the RIA vials and capped. All the samples were kept in a vibramax centrifuge for 10 min at 2500rpm. All the samples were centrifuged for 5min at 10000 rpm in a refrigerator centrifuge. 0.4ml of organic layer was transferred into respective labelled RIA vials. The organic layer was dried in a nitrogen evaporator at 400C. The dried residue was reconstituted with 0.1ml of mobile phase and vortexed. Reconstituted samples were transferred in to respectively labelled auto injection vials. 5µl of the above was then injected in to LCMS/MS system using the chromatographic condition described below.

Table 2: Chromatographic condition of LC-MS/MS

Apparatus Parameters	Specification
Apparatus	LC-MS/MS (SHIMADZU)
Column	C 18 column (3x50mm)
Mobile Phase	2M ammonium acetate :Methanol (20:80) v/v(Binary flow)
Temperature	40°C
Injection volume	5µl
Flow rate	0.2ml/min
Run time	3 minutes
Sample cooling temperature	10°C

Calculation of the concentration

The concentration of the unknown was calculated from the following method using regression analysis of spiked plasma calibration standard with the reciprocal of the square of the drug concentration as

Weighing factor (1/concentration X concentration)

$$y = mx + b$$

Where

X – Concentration Analyte

m – Slope of the calibration curve

Y – Peak area ratio of analyte to internal standard (IS)

b – Y-axis intercept of the calibration curve

Procedure for Data Analysis

Pharmacokinetic parameters were estimated using model-independent methods, WINNOLIN Scientific Software, Statistical Consultant, and Apex, NC, USA), nonlinear least squares regression and computer programs. The noncompartmental Analysis for extra vascular administration in WINNOLIN was used to measure the area under losartan concentration time curve (AUC) for a period of 1440 minutes (t= 1440 minutes), the area under the first moment of the curve (AUMC), the mean residence time (MRT = AUMC/AUC). The apparent total clearance (Cl/F) was calculated using noncompartmental equations where, CI/ i7 = (dose/AUC). The Annova software was used to determine statistically significant differences ($P<0.05$) of *in vivo* data.

Statistical analysis

One way analysis of variance (ANOVA) using Dunnett multiple comparison test on computer program Graphpad Instat 3 was used.

RESULT AND DISCUSSION

***In vivo* evaluation studies in rabbit.**

The ability of pulsatile tablets as a drug delivery system to release drugs in a predetermined time release manner was investigated in New Zealand rabbits after oral administrations was investigated.

Losartan was used as the marker drug. The pulsatile drug delivery system tablet prepared under laboratory conditions release the drug in –vitro in a uniform and reliable manner, these data indicated that the device should be suitable for in-vivo evaluation in animals. Mean plasma drug concentration curve v/s time for both the groups of rabbits was studied for comparing various pharmacokinetic parameters. Maximum drug plasma concentration (Cmax) and the time to maximum value (T max) were obtained directly from the drug plasma profile for each animal following administration of all the three above mentioned dosage formulations. The AUC 0-24 for animals Group A given pure drug losartan was found to be19863219.9 nanograms/ml/hr whereas the AUC 0-24 for animals administered with pulsatile release tablet, the AUC 0-24 was found to be 24379126.35nanograms/ml/hr. MRT is defined as the mean time for the intact drug molecule to transit through the body and involved acomposite of all kinetic processes including release from the dosage form, drug absorption into the body and drug disposition. MRT can be used in a comparative way to evaluate the *in vivo* performance of a pulsatile release dosage form. Therefore, the increase in the MRT from 2.014 to 8 hours following Losartan pure drug and pulsatile drug, respectively, was mainly due to the change in drug release and elimination. The average tmax values were found to be 2.0 ± 0.78 hr (120 mins), and 8.0 ±0.95hr (480 mins) for pure drug losartan and pulsatile drug respectively. Pure drug formulation showed low value of tmax (2hours) which indicates faster absorption of the drug as compared to pulsatile drug formulation. As per the summary of pharmacokinetic parameters as given in Table no 3 one can predict that pure drug Losartan showed pattern of drug absorption and pulsatile drug formulation showed a lag time of 3 hours before finally showing maximum concentration (Cmax) at 8 hours, which correlated with the in-vitro release (8 hours) One way analysis of variance (ANOVA) using Dunnett multiple comparison test on computer program Graphpad Instat 3 was used, the differences were considered significant at p value equal or less than 0.05($p \leq 0.05$).

Table 3: Summary of Pharmacokinetic Parameter

Pharmacokinetic Parameter	Group A	Group B
AUC (0-24) (nanogram/ml/hr)	19863219.9 ± 0.84	24379126.35 ± 2.35
AUC (t-∞) (nanogram/ml/hr)	19863332.2 ± 0.55	24379234.45 ± 0.87
AUMC (0-24) (nanogram/ml/hr)	1194385860 ± 1.85	13481239890.6 ± 0.86
Cmax, ng/ml	58734.612 ± 0.34	54632.231 ± 0.43
Tmax,hr	2.0 ± 0.78 hr (120 mins)	8.0 ± 0.95hr (480 mins)
T1/2 hr	1.9 hours	7.8 ± 0.45hours
MRT (hrs)	2.014 ± 2.34hours	9.2 ± 4.85hours

SUMMARY AND CONCLUSION

From the above work it can be concluded that the developed LC-MS/MS method was highly sensitive and suitable for the detection of Losartan in plasma in concentrations as low as 0.5 nanogram/ ml. In conclusion, pulsatile drug release over a period of 4-12 hrs, consistent with requirements for chronopharmaceutical drug delivery, was achieved from a bi layered tablet to modulate the drug release time in accordance with chronotherapeutic objectives.

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