IN-VIVO STUDIES OF PULSATILE-CAPSULAR, MARKETED CONVENTIONAL AND ACTIVE PHARMACEUTICAL DRUG DELIVERY OF THEOPHYLLINE BY LC-MS/MS METHOD

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Summary

Chronopharmacokinetic studies have been reported for many drugs in an attempt to explain chronopharmacological phenomena and demonstrate that the time of administration is a possible factor of variation in the pharmacokinetics of a drug. Nocturnal asthma is defined as any sleep related worsening of reversible airway disease. Symptoms generally include shortness of breath or coughing and wheezing at night. Approximately 80 percent of severe asthmatic attacks occur between midnight and 8 a.m. Systemic absorption from the colon can also be used as a mean of achieving chronotherapy for diseases that are affected by circadian rhythms such as asthma, angina and arthritis. Theophylline (TPH), one of the commonly used drugs in the treatment of nocturnal asthma, was chosen as a model drug in this study. It is a nonselective phosphodiesterase inhibitor, also a bronchodilator which enhances the respiratory muscle function and mucociliary clearance; it also acts in the central nervous system to enhance ventilation. The dosage form of 400 mg each were administered to 3 groups of white New Zealand rabbits (n=6) following cross over design pattern and the plasma levels were measured using LC-MS/MS method. The comparison of the plasma time curves of the dosage forms showed that each dosage form caused significant differences in the drug plasma levels. The plasma drug profiles of active pharmaceutical ingredient and marketed conventional tablet of theophylline showed nearly similar pattern of drug release, whereas the pulsatile capsular formulation prepared in the laboratory managed to show some lag phase initially before releasing the drug. The pulsatile drug delivery capsule showed maximum time (Tmax) at the 8th hour in comparison to active ingredient which showed plasma peak in the range of 2-3 hours.

Keywords: Theophylline, pulsatile, In-vivo studies, Cross-over design, LC-MS/MS

Introduction

Bioavailability studies are usually evaluated by a single-dose, crossover design, comparing equal doses of the test and reference products in fasted, adult, healthy subjects[1]. The use of healthy subjects may give false misleading results since drugs are usually given to patients with diseases. On the other hand, applying the fasting state excludes the effect of food on drugs' absorption. Currently, three different studies may be required for solid oral dosage forms, including (1) a fasting study, (2) a food intervention study, and/or (3) a multiple-dose (steady-state) study and other study designs have been proposed by the FDA in 1997 to consider the performance of individual bioequivalence studies using a replicate design and a two-way crossover food intervention study[2]. The term "Chrono" pertains to time and "Biology" is the science of life, thus, chronobiology concerns with the observation of every metabolic event goes through rhythmic changes in time that can be measured from seconds to seasons[3]. Applying this science could help in prevention and/or early diagnosis and treatment of diseases, and consequently, reduction in the overall health care costs. Chronotherapeutics is defined as a treatment system where the in vivo drug availability has been timed in accordance to cyclic rhythms of drug related biological phenomena to create maximum benefit minimizing harm. Different pharmacokinetics constraints of time like elimination rate, peak concentration, volume of distribution, and AUC of a number of drugs are affected by circadian rhythms[4]. The introduction of a new system that can benefit from the known circadian rhythms of the disease and overcome some of the shortcoming of the current available treatment, this can achieved by modifying the release of the drug to provide higher drug levels in the period when the disease intensity is peaking (2-6 am). The oral colon drug delivery systems can be utilized to provide beneficial attributes in the treatment of nocturnal asthma[5]. They exhibit lag time of absorption followed by relatively rapid drug absorption for extended period due the larger colon residence time. The design of the colonic formula can tremendously affect the drug release profile. Nocturnal asthma is associated with critical symptoms and urgent need for proper medications[6]. The onset of nocturnal asthmatic attacks is rare in the first part of night, 80% of asthmatic attacks occur between midnight and 8 a.m., and deaths from asthma are more common during these hours. In a study of asthma mortality, 79% of the patients who died had a disturbed sleep before the death; this may lead to leaving the patient unprotected against the worse events of nocturnal asthma[7]. Thus, a smart drug delivery that is administrated before sleep and maintains high blood levels for longer period (from midnight to 8 am in the morning, during which maximum intensity of the disease occurs) could be very much beneficial for proper management of nocturnal asthma[8-9]. Systemic absorption from the colon can also be used as a mean of achieving chronotherapy for diseases that are affected by circadian rhythms such as nocturnal asthma[10]. If drug absorption throughout the gastrointestinal tract is not limited, then pulsatile drug delivery might be a suitable alternative to repeated dosing. This might be especially useful if peak plasma levels are desirable in the night time or the early morning hours. Pulsatile drug delivery system have a number of advantages like to maintain constant plasma drug level, time of administration (during morning hours), would be ideal. Same is true for preventing asthmatic attacks in the middle of the night and the morning stiffness typical of people suffering from arthritis[11-13]. Drugs that produce biological tolerance demand for a system that will prevent their continuous presence at the biophase as this tends to reduce their therapeutic effect. The objective of this study was to investigate differences in the pharmacokinetic patterns between a pulsatile drug delivery system using a pulsatile capsule, an immediate release delivery (conventional) and pure drug

theophylline all of same dose[14-18]. Theophylline was chosen as a model drug because of its high solubility and high permeability pattern throughout the GI tract. The dosage forms were administered to three group's containing six of white New Zealand rabbits each, and the plasma levels were measured using LC-MS/MS. Cross-over design pattern was used for the above studies[19]. Pharmacokinetic parameters were determined for each dosage form and compared. Fluctuations in the plasma time curves over the observation period indicated that physiological factors like motility have an influence on the drug absorption[20]. The comparison of the plasma time curves of the dosage forms showed that each dosage form caused significant differences in the drug plasma levels. The pulsatile drug delivery capsule caused two defined C(max) values for each dose between 1-1.75 and 2.5-3.5h. Implications for the use of a pulsatile drug delivery device for chronopharmacotherapy are discussed. Pulsatile drug delivery offers a promising way for chronopharmacotherapy if the time of administration and pulse time are adjusted to the circadian pattern[21-23].

Materials and Methods

Materials

The following chemicals were obtained from commercial suppliers and used as received: Pure Theophylline (Cipla, Bangalore, India), Phenacetine-AR grade, Ethyl acetate-AR grade, Methanol-HPLC grade, Ammonium acetate-AR grade, MilliQwater-HPLC grade (S D Fine Chemicals ltd, India), Sodium Heparin , 0.45µm nylon 6,6 membrane filter, Micro tips (200µl-1000µl), Auto injection vials, RIA vials and Micro tips (200µl-1000µl).

Methods

Experimental animals

In-vivo studies were carried out after pertaining Animal Ethical Clearance from CPSCEA committee using eighteen male New Zealand rabbits weighing between 2.0 and 2.5 kgs, either sex, all of them were healthy and used for the first time as experimental animals. All dogs were fasted for about 24 hours prior to drug administration and continued fasting until 5 hours post-dose, with water allowed during the study. During the experimental period, rabbits were placed in a normal cage without using a restrainer stand.

Study design

Three-way cross-over design was applied in this study. The rabbits were divided into three groups, each contain six each (n=6). Each group received one of the three treatments in each phase. A washout period of at least one week was allowed between treatments. The treatments were given by normal stomach tube by 100 ml water. Five-ml blood samples were collected, in heparinized evacuated plastic tubes by using a 5 ml syringe for each sample which is collected

from left lobular vein, at 0 (predose), 30, 60, 120, 180, 240, 360, 480, 600, 720, 1200 and 1440 minutes from the time of administration. The plasma was immediately separated by aspiration after centrifugation at 5000 rpm for 5 minutes and frozen at -20 ºC until analysed.

Dosage forms

Active pharmaceutical drug theophylline , Conventional marketed theophylline (400mg) and Insoluble capsular pulsatile drug release of theophylline (400 mg) sealed with a polymer plug prepared in the laboratory conditions and chosen on the basis of drug content , lag time achieved , in-vitro release studies and stability conditions were chosen as dosage forms for administration.

Method development by LC-MS/MS

The standard stock solution for theophylline was prepared by weighing 2mg of theophylline working standard 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 theophylline. The above final concentration for theophylline was corrected for accounting for its potency and the actual amount weighed. It was then stored in refrigerator or cooling cabinet. The Internal standard (IS) stock solution was prepared by weighing about 2mg of internal standard (Phenacetin) 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.

Sample preparation

All samples of one or more periods of one or more subjects 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 in to 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 for 10 min at 2500rpm. All the samples were centrifuged for 5min at 10000rpm 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 40^0C. 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.

Chromatographic Conditions

Column composed of hypurity advance C18 Column (3x50mm), Mobile Phase composed of 2mM Ammonium acetate: Methanol:: 20:80V/V (Binary Flow) mixture, the injection volume was about 5µl, the Flow rate was about 0.2ml/min without splitter (Binary Flow), and the Run time was fixed at 3 minutes, the Column oven temperature was about 40^0C and the sample cooler temperature was fixed at 10^0C.

Pharmacokinetic analysis

The pharmacokinetic parameters were calculated from the plasma level data obtained from the individual rabbit and presented as mean ± SD. A plot of the mean plasma concentration versus time has been constructed for each of the three treatments. From the data of plasma concentration at each sample time, the maximum plasma concentration (C_{max}, µg/ ml); and the corresponding time for the maximum plasma concentration (t_{max}, h) were directly determined for the three treatment in each individual animal. The area under the plasma concentration- time curve from time zero to 24 h (AUC_{0-24}, µg. h/ml) has been obtained by applying the trapezoidal rule. The $(AUC)_{24-\infty}$ was estimated from the following equation:

$$(AUC)_{t-\infty} = C_{pt}/K_{el}$$

Where:
C_p is the least measurable concentration at time t. K_{el} is the elimination rate constant calculated utilizing the least- squares regression analysis. The area under the first moment curve (AUMC 0-24, µg. h^2 /ml) was obtained by applying the trapezoidal rule to data of the plasma concentration multiplied by the corresponding time versus time for each of the tested treatments. The AUMC 0-24 was estimated using the following equation:

$$AUMC_{24-\infty} = t\,C_{pt}/K_{el} + C_{pt}/K_{el2}$$

The $AUMC_{0-\infty}$ was obtained directly by adding the $AUMC_{24-\infty}$ to $AUMC_{0-24}$.

The mean residence time (MRT, h) which is noncompartmental pharmacokinetic parameter was obtained from the following equation:

$$MRT = AUMC_{0-\infty} / AUC_{0-\infty}$$

Statistical analysis

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

Results and discussion

It is clear from **Figure no 1** that good peak separation between theophylline (TPH) under the applied LCMS/MS conditions was detected with reasonable retention times of 1.6 to 1.9 minutes, respectively. No interference was noticed, with blank rabbit plasma.

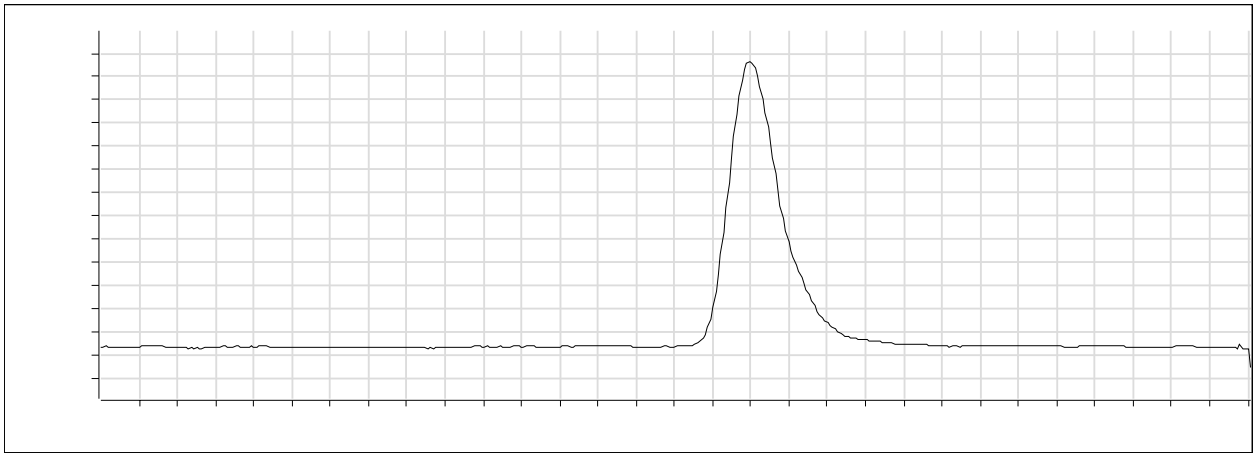

Figure no 1; Chromatogram of plasma concentration of theophylline

Figure no 2 as shown below shows standard calibration curve that was obtained by plotting the mean peak height ratios of TPH to Internal standard (IS) against the corresponding TPH concentrations in the range from 0 to 500 ng/ml. The precision

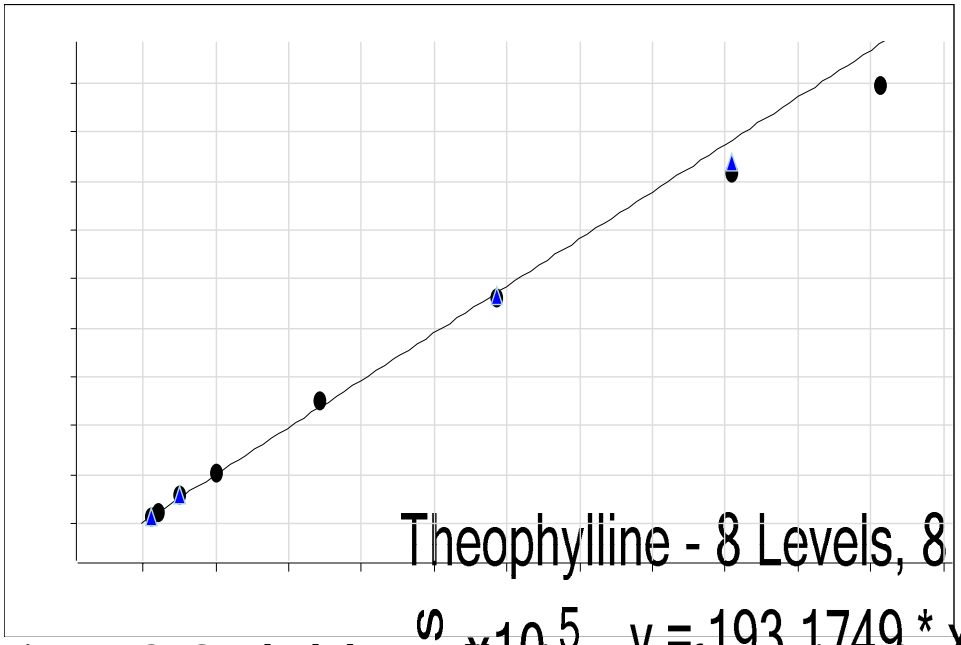

Figure no 2 – Standard plasma calibration curve of TPH using LC-MS/MS

The precision of using the calibration curve for calculating the TPH concentration in plasma was tested by analysing three known concentrations representing low (1 ng/ml), medium (2500 ng/ml), and high (5000 ng/ml). The % recovery ranged from 99.711 to 105.037 and the RSD % was in the range of 4.151 to 7.210. These results are in accordance with the assay validation requirements and revealed the suitability of the method for the determination of TPH in the unknown samples. The Bias % for three concentrations was in the range of (from 0.288 to 5.038) indicating high precision in using the constructed calibration curve for the determination of unknown TPH concentrations in rabbit plasma in the range of 0 - 5000 ng/ml. The linear regression analysis revealed a straight line with the following equation: Y=193.1749x+2474.9578, and a correlation coefficient of r = 0.99242330. This proves a very good linearity over the range of concentrations 0- 5000 ng/ml of TPH in plasma. The retention time for plasma drug concentration of theophylline was found to be 1.707 as shown below in **Figure no 3**

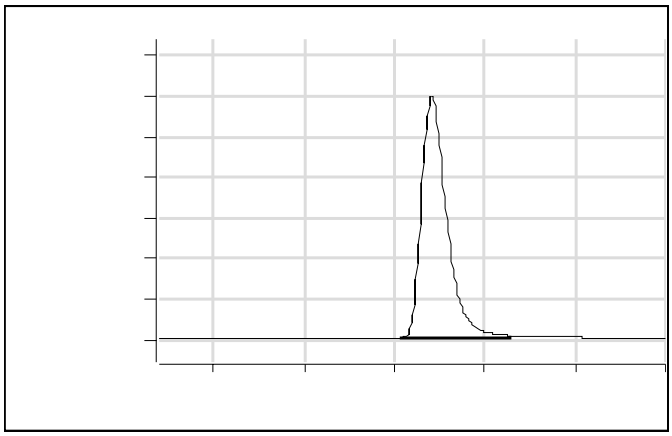

Figure no 3: Retention time of plasma drug concentration of theophylline

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 theophylline was found to be 19863219.9 nanograms/ml/hr and animals given marketed conventional tablet of theophylline (400 mg), AUC_{0-24} was found to be 23885079.86 nanograms/ml/hr whereas the AUC_{0-24} for animals administered with pulsatile release AUC_{0-24} was found to be 24379126.35 nanograms/ml/hr. MRT is defined as the mean time for the intact drag molecule to transit through the body and involved a composite 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 theophylline pure drug and pulsatile drug, respectively, was mainly due to the change in drug release and elimination. The average t_{max} values were found to be 3 $\pm$ 0.12hr (180 mins), 2.0 $\pm$ 0.78 hr (120 mins), and 8.0 $\pm$ 0.95hr (480 mins) for marketed conventional, pure drug theophylline and pulsatile drug respectively. Pure drug formulation showed low value of t_{max} (2 hours) 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 1** below one can predict that pure drug theophylline and marketed conventional formulation showed almost similar 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).

Pharmacokinetic Parameters	***Group A (Marketed Conventional)***	***Group B (Pure drug)***	***Group C (Pulsatile)***
AUC 0-24 (nanograms/ml/hr)	23885079.86 $\pm$ 1.24	19863219.9 $\pm$ 0.84	24379126.35 $\pm$ 2.35
(AUC) $_{t-\infty}$ (nanograms/ml/hr)	23885130.67 $\pm$ 0.46	19863332.2 $\pm$ 0.55	24379234.45 $\pm$ 0.87
AUMC $_{0-24}$ (nanograms/ml/hr^2)	118609396537 $\pm$ 0.23	1194385860 $\pm$ 1.85	13481239890.6 $\pm$ 0.86
Cmax, ng/ ml	56699.516 $\pm$ 0.81	58734.612 $\pm$ 0.34	54632.231 $\pm$ 0.43
t_{max},hr	3 $\pm$ 0.12hr (180 mins)	2.0 $\pm$ 0.78 hr (120 mins)	8.0 $\pm$ 0.95hr (480 mins)
$t_{1/2, hr}$	2.3 $\pm$ 0.78 hours	1.9 hours	7.8 $\pm$ 0.45hours
$K_{el(hr^{-1})}$	0.11 $\pm$ 0.65	0.14 $\pm$ 1.83	0.21 $\pm$ 0.55
MRT(hrs)	2.9 $\pm$ 3.33 hours	2.014 $\pm$ 2.34hours	9.2 $\pm$ 4.85hours

Table no 1: Summarized pharmacokinetic parameters of all the dosage forms

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 ($p \leq 0.05$).

Conclusion

LCMS/MS technology is a very significant in detecting minutest plasma concentration in nanogram level maintaining its accuracy , efficiency and precision. In conclusion, pulsatile drug release over a period of 4-12 hrs, consistent with requirements for chronopharmaceutical drug delivery, was achieved from a insoluble gelatin capsule where drug was sealed within the capsule body by means of an erodible plug.

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