Digest Journal of Nanomaterials and Biostructures Vol. 5, No 2, April-June 2010, p. 403 – 409

BIOANALYTICAL METHOD DEVELOPMENT AND COMPARATIVE BIOAVAILABILITY STUDY OF CLOBAZAM TABLETS IN ALBINO RATS PLASMA USING RP-HPLC METHOD

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A rapid and specific bioanalytical method was developed for clobazam (100 mg) in albino rat plasma using ibuprofen as an internal standard (IS) and bioequivalence study has also been carried out on two marketed formulation. The sample was prepared by liquid–liquid extraction with acetonitrile yielding almost near 100% recoveries of clobazam. Chromatographic separation was achieved with a RP-HPLC C_{18} column, using a mixture of a water (pH 3.5, adjusted with orthophosphoric acid): acetonitrile (55:45 v/v) and was binary eluted at flow rate of 1.5mL min^{-1}at a detection wavelength of 220 nm. The calibration curves were linear ($r^2 > 0.999$) in the concentration range of 5-100 ng ml^{-1}. The lower limit of quantification was 3 ng ml^{-1} for two compounds studied.The extraction effiecency for the clobazam was more than 99.99%. The within and between day precisions in the measurement of four tested concentrations were in the range of 0.89–9.1% and 2.1–10.1% R.S.D., respectively. The developed procedure was applied to assess the pharmacokinetics of clobazam following administration of 5mg/kg oral dose of clobazam to healthy albino rats. The described method was established as a rapid analytical tool in a pharmacokinetic study requiring short retention time, high precision, sensitivity and small volumes of plasma for analysis of benzodiazepine derivatives.

(Received April 27, 2010; accepted May 5, 2010)

Keywords: Clobazam, AUC, Ibuprofen, RP-HPLC, Bioequivalence.

1. Introduction

Clobazam (CLB) is a 1,5-benzodiazepine (fig1) with anxiolytic and anticonvulsant properties and is used for sedation and as an antiepileptic drug, presenting some advantages over 1,4-benzodiazepines [1]. In other applications clobazam is used as a covering drug when there is a change in therapy. The drug's action is very quick usually effective within a couple of hours but no longer than a few days [2] and the monitoring of the drug's haematic levels is of great clinical interest in order to determine its correct use. clobazam (CLB) has been administered to patients before cardiopulmonary bypass surgery [3] and we were interested in the effect of such a procedure on CLB pharmacokinetics. Thus a sensitive method for the determined CLB in plasma was necessary. Several techniques have been reported for CLB quantification, using gas chromatography (GC) [4-6] and High-performance liquid chromatography (HPLC) [7-10]. These techniques have been reviewed. HPLC methods for CLB and other benzodiazepines can be sensitive determination in urine has been reported. This paper presents a simple and sensitive procedure for CLB determination in rat plasma suitable for therapeutic drug monitoring, pharmacokinetic and bioavailability studies as well as for toxicological purposes.

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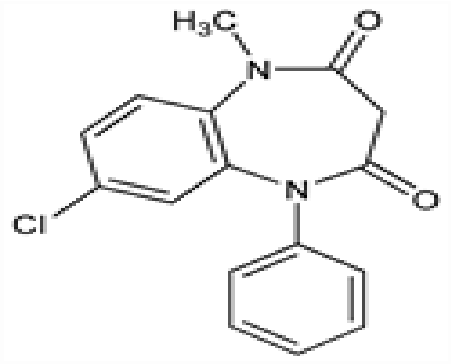

Fig.1. CLOBAZAM

2. Experimental

2.1 Instrumentation

The HPLC system (Shimadzu, Japan) consisted of a LC-10AT VP pump, a SPD-10AVP, PDA detector, a Phenomenex Luna C18 (250mmX4.6mm, 5µm) column, a Phenomenex, HPLC guard cartridge system and a Class cbm-20A/20 Alite software was used for study.

2.2 Chemicals

The chemical and all reagents used were ortho phoporic acid and sodium hydroxide were of analytical grade; acetonitrile, and methanol were of HPLC grade. Clobazam (7-Chloro-1-methyl-5-phenyl 1H-1,5-benzodiazepine-2,4- [3H,5H] dione) and ibuprofen (*RS*)-2-(4-(2-methylpropyl) phenyl) propanoic acid were provided free of charge by lake chemical. Pvt.Ltd, New Delhi, India. The columns packed with 1 g of celite, were supplied from Shimadzu, Japan.

2.3 Chromatograpic condition

The chromatographic analysis was performed by using a mobile phase composed of water (pH 3.5, adjusted with orthophosphoric acid): acetonitrile (55:45 v/v) and was binary eluted at flow rate of 1.5mL min^{-1}. The chromatogram was monitored with UV detection at wavelength of 220 nm using the internal standard Ibuprofen

2.4 Preparation of standard solutions

Stock solutions of all the compounds (1 mg/ml) were prepared in acetonitrile and were stable for 3 months when stored at 4^0C. Working solutions were prepared daily from these stock solutions by dilution with acetonitrile [11].

2.5 Sample preparation and storage

The plasma samples were prepared by liquid–liquid extraction (LLE). The conditions consisted of mixing 0.1ml of plasma with 0.05 ml ibuprofen as internal standard (from the stock 6000 ng/ml) in a 2 ml Eppendorf polypropylene tube and then extracting with 1.5 ml of acetonitrile. After vertical agitation (1 min) and centrifugation 5000 rpm, 15 min), the upper organic layer was injected on to the HPLC system for determination [12]. Fourteen rats were included in this study. The study protocol was approved by the Ethics Committee of KMCH College of pharmacy, Tamilnadu, India. Rats were not allowed to take any other medication for 2 weeks before and throughout the study. The Rats received two marketd tablet formulatrions Frisium (Aventis, UK), lobazam (sun pharma) at the dose of 5mg/kg body weight [13] after an

overnight fast in single bioequivalence study. Intake of food was delayed for 3 h after medication. Blood samples were collected from the rat tail at predetermined intervals from each rat and plasma samples were stored at -20^0C until analysis.

2.6 Preparation of calibration standards

Starting from pooled stock solution of clobazam 1 mg/ml in acetonitrile, standards were prepared using pooled rat drug free plasma obtained from healthy rat as diluent. The calibration curve was performed with standards of the final concentrations of 5, 10, 25, 50 and 100 ng/ml in rat plasma. Working solution of ibuprofen (3000 ng/ml in acetonitrile) was prepared daily by dilution of stock solution (1 mg/ml in acetonitrile).

2.7 Accuracy, precision, limit of quantification (LOQ) and recovery

Accuracy, between and within-day precisions of the method were determined for each compound according to FDA guidance for bioanalytical method validation [FDA Guidance]. Three replicate spiked plama samples were assayed between and withinday at four different concentrations (5, 10, 25, 50 and 100 ng/ml) for each analyte. The concentrations were calculated using calibration curves prepared and analyzed in the same run. Accuracy was calculated as deviation of the mean from the nominal concentration. Between and within day precision were expressed as the relative standard deviation of each calculated concentration. For the concentration to be accepted as LOQ the percent deviation from the nominal concentration (accuracy) and the relative standard deviation has to be ±20% and less than 20%, respectively, considering at least five times the response compared to blank response and the LOQ found was with in the range Average recovery of each compound was determined by comparing AUC obtained after injection of the samples with those achieved by direct injection of the same amount of drug in distilled water at different concentrations (three samples for each concentration level).

2.8 Calculation of pharmacokinetic parameters

Plasma concentration time curves of CLB were evaluated by non compartmental analysis. Maximum plasma concentration C_{max} and the time t_{max} were obtained directly from the individual plasma concentration versus time curves. The terminal half-life, $t_{1/2}$ was obtained from loglinear regression analysis of the plasma concentration time curves in the terminal phase. The area under plasma concentrationtime curve up to last quantifiable plasma concentration (AUC_{lqc}) was determined according to the linear trapezoidal method [14].

3. Results and discussion

3.1 Selectivity and chromatography

The separation achieved using the experimental conditions of the present assay for clobazam are presented in (fig2) Selectivity was indicated by absence of any endogenous interference at retention times of peak of interest as evaluated by chromatograms of control rat plasma and plasma spiked with clobazam. Retention times for CLB and internal standard were 6.0 and 10.4 min, and their relative R.S.Ds (%) calculated from 25 consecutive injections were 6.0 and 10.4 respectively. No change in column efficiency and back pressure was observed over whole study time.

3.2 Linearity

Ten point calibration curves for CLB on separate days were linear over the concentration range of 5–100ngml^{-1}. The equations for means (n = 3) of six standard curves are: for CLB y=5.9126x-0.8777 (r^2=0.999) (fig 3).

3.3 Limit of quantification

LOQs as defined previously were 5 $ngml^{-1}$ for each compound. The LOQ values for two analytes are reported (Table1).

3.4 Recovery, accuracy and precision

The results from the validation of the method in rat plasma are listed in table 1. The method proved to be accurate and precise: accuracy at four concentration levels ranged from 83.4 to 100.7% for all compounds. The within and between day precision ranged from 0.890 to 9.1% and 2.1 to 10.1%, respectively for all analytes. The absolute recoveries ranged from 93.5 to 102.3%.

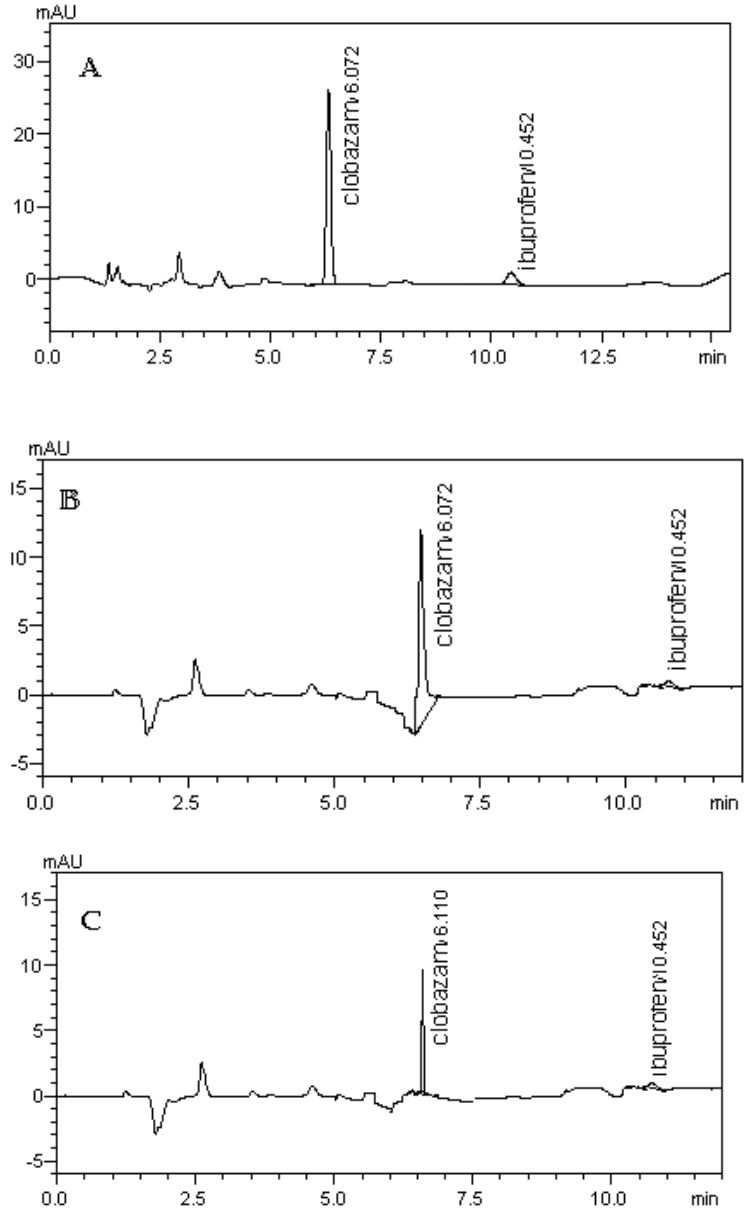

Fig. 2. (A) Chromatograms of spiked human plasma with 50ngclobazam and 500ng ibuprofen, (B) Chromatograms of clobazam (lobazam) in plasma at T_{max} (C LB = 20.545 ng/ml and ibuprofen = 500 ng/ml). (C) Chromatograms of clobazam (frisium) in plasma at T_{max} (CLB = 24.862 ng/ml and ibuprofen = 500 ng/ml).

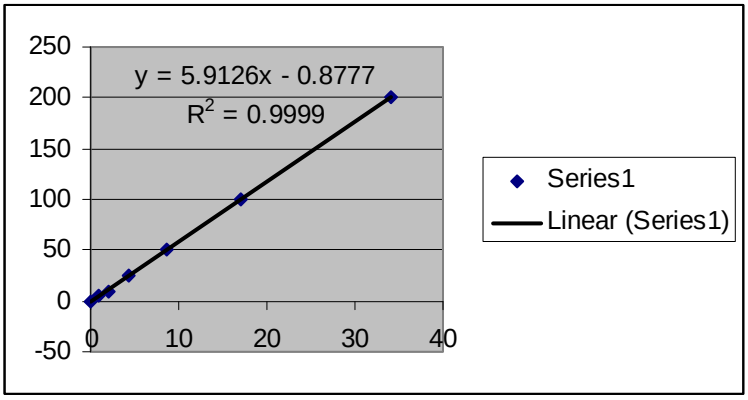

Fig. 3. Calibration curve of clobazam

Limit of quantification

LOQs as defined previously were 5 $ngml^{-1}$ for each compound. The LOQ values for two analytes are reported (Table1).

Table1: Limit of quantitation (LOQ) for clobazam

Brands	Concentration($ngml^{-1}$)	BetweendayR.S.D(%)	Accuracy
lobazam	3.688	5.8	88.1
Frisium	3.034	2.9	92.3

Recovery, accuracy and precision

The results from the validation of the method in rat plasma are listed in table 2. The method proved to be accurate and precise: accuracy at four concentration levels ranged from 83.4 to 100.7% for all compounds. The within and between day precision ranged from 0.890 to 9.1% and 2.1 to 10.1%, respectively for all analytes. The absolute recoveries ranged from 93.5 to 102.3%.

Table 2: Between- and within-day variability, accuracy, and recovery for determination of lobazam and frisium. (CLB)

Concentration(ng/ml) Clobazam	**Between-day variability(n=3)**		**Within-day variability(n=3)**		**Recovery(n=3)**	
	R.S.D (%)	**Accuracy (%)**	**R.S.D (%)**	**Accuracy (%)**	**%**	**R.S.D (%)**
lobazam						
5	9.9	83.4	9.1	81.9	102.3	4.0
25	8.8	97.5	5.7	94.6	93.6	2.9
50	10.1	98.3	3.3	94.8	99.8	0.9
100	8.8	93.2	0.4	91.4	93.8	3.3
Frisium						
5	4.5	92.3	12.6	103.8	93.5	6.3
25	2.1	102.3	7.6	100.2	95	3.4
50	8.6	100.7	0.9	99.5	98.0	2.2
100	5.5	94.4	1.5	93.8	100.2	1.9

Table 3. Pharmacokinetic data (mean±S.D.) obtained from albino rats following oral administration of 10 mg clobazam (n = 14)

Brand	T_{max}(h)	C_{max}(ngml^{-1})	AUC_{iqc}(nghml^{-1})	$T_{1/2}$(h)
Indian	1.00	20.545	38.638	0.388
MNC	1.00	24.862	54.4458	0.0802

4. Conclusions

To apply the developed and validated method, the pharmacokinetics of clobazam was assessed in 14 albino rats. The pharmacokinetic parameters of clobazam derived by non-compartmental analysis are summarized in table 3. The described method was established as a rapid analytical tool in a pharmacokinetic study requiring short retention time, high precision, sensitivity and small volumes of plasma for analysis. The parameters of the assay obtained in the course of validation processes presented above in the results section were considered satisfactory for its clinical application. A simple analytical procedure based on one-step extraction and a total run time of 11 min allows the possibility of determination some 18 samples a day. The procedure originally developed for clobazam was found to be effective for benzodiazepine derivatives in general and it seems that the method may be easily adapted for such drug determinations.

Acknowledgement

The authors are thankful to Chairman Dr. Nalla G. Palaniswami& Dr. Thavamani D. Palanisami, Kovai medical center research and educational trust, Coimbatore, for providing facilities and laboratories.

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