Study of Pharmacokinetics and Comparative Bioavailability of Nefopam 30 mg Tablets in Twelve Fasting Healthy Pakistani Male Young Subjects: Single-Dose, Randomized, Two-Period, Two-Treatment and Two-Way Cross-Over Design

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Key Words

Nefopam • Bioavailability • Pharmacokinetics • Plasma • LC/MS

Conclusion: The results showed that both formulations possessed almost the same relative bioavailability and pharmacokinetic parameters.

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Abstract

Objective: To study the pharmacokinetics and comparative bioavailability of nefopam tablets (Acupan®). **Subjects and Methods:** Experimentation of this study was based on a single-dose, two-sequence, cross-over randomized design using 12 fasting healthy Pakistani male young subjects. This validated LC/MS method was applied to a pharmacokinetic and bioavailability study in 12 fasting healthy Pakistani male subjects from the blood samples taken up to 24 h after an oral dose of one tablet of 30 mg nefopam in a double-blind, randomized, cross-over design. **Results:** The mean maximum plasma concentration (C_{max}) for the reference formulation was 60.71 ± 2.36 ng/ml ($\pm$ SEM) and for test formulation 60.46 ± 1.30 ng/ml ($\pm$ SEM). The mean time to reach maximum plasma concentration (T_{max}) values of reference and test formulations was 1.63 ± 0.13 h ($\pm$ SEM) and 1.83 ± 0.07 h ($\pm$ SEM), respectively. The mean $\pm$ SEM values of $AUC_{0-\infty}$ for the reference and test formulations were 293.01 ± 16.09 ng·h/ml and 307.53 ± 8.99 ng·h/ml, respectively.

Introduction

Nefopam hydrochloride, 5-methyl-1-phenyl-1,3,4,6-tetrahydro-2,5-benzoxazocine, is a cyclic analogue of diphenhydramine and an analgesic which is active both orally and parenterally. Nefopam is a centrally acting analgesic which does not compromise ventilation and is minimally sedating. Its efficacy relies on medullar and/or supramedullar mechanisms. Its fast onset of action is an advantage, especially in the treatment of postoperative pain [1].

The pharmacokinetics and comparative bioavailability of nefopam has been reported [2]. When administered orally, its bioavailability is low most likely due to first-pass metabolism, its peak plasma concentration is achieved within 1–3 h and the terminal half-life is 4.0–5.1 h [2]. Nefopam is mainly excreted via urinary tract.

As of today in Pakistan, no study assessing the pharmacokinetics and bioavailability of nefopam has yet been conducted. Hence, the objective of this study was to determine the pharmacokinetics and comparative bioavailability of nefopam in 12 fasting healthy Pakistani male young subjects.

Subjects and Methods

Study Design and Subject Selection

Twelve male Pakistani subjects 21–28 years of age with a body weight ranging from 50 to 70 kg, and considered being in good health based on medical history, physical examination and laboratory test values (hematology, serum chemistry, urine analysis and creatinine levels) were enrolled in the study which was conducted in fasting state. The study design was a single-dose, randomized, two-period, two-treatment and two-way cross-over and carried out at the Bioequivalence Center (The Islamia University of Bahawalpur, Pakistan). This study was approved by the Board of Advanced Studies and Research, The Islamia University of Bahawalpur, Pakistan and was carried out in compliance with the Helsinki rules [3] for human use in research and the Principles of Good Clinical Practice [4]. Subjects were not taking any medication for at least two weeks before the study began to avoid any interference. All subjects were informed of the aim of study. Informed written consent was obtained from each volunteer before the start of study.

Study Drug Administration

All subjects received a single dose of both test (Inza® tablets 30 mg; Test, Mediceena Pharma, Pakistan) and reference formulations (Acupan® tablets 30 mg; Reference, 3M Pharmaceuticals, UK) in random order based on computer-generated random number tables. The two study periods were separated by a 1-week washout period. Subjects were hospitalized at 7 p.m. on each night before study drug administration and received a standardized dinner (920 ± 20 kcal; 65% from carbohydrate, 20% from protein, and 15% from fat) [5] in the hospital. Based on their medical history, they were not allergic to the nefopam and were nonsmokers and did not drink alcohol. Due to the cooperation from the subjects, it was easy to obtain reports of any side effect and to retain them in the sampling area for 12 h prior to and 12 h after the sampling. A physician and two nurses looked after the whole sampling process. They monitored the subjects for their vital signs: blood pressure, respiration rate and temperature. An unblinded physician (Dr. Usman Ghani) met with the subjects to monitor adverse effects occurring during the study period. The subjects were advised to fast overnight prior to treatment. The subjects were provided with breakfast consisting of 2-egg omelets, 4 pieces of toast and fruit juice 4 h after dosing. They were also provided with a standardized lunch [6] at 10 h after the drug administration, keeping in mind that all the subjects receive a similar type of foodstuffs. Each volunteer was given single dose of nefopam 30-mg tablet (test or reference) orally with 250 ml of tap water. The following week, after a wash-out period of 1 week, the subjects were given the test or reference different from the previous week. All subjects completed both treatment periods. No protocol

deviations were noted. Beverages containing xanthine derivatives or alcohol, intense physical activity, and smoking were not allowed over the course of the study. All subjects were under continuous medical supervision at the sampling site throughout the study.

Blood Sample Collection

A 22-gauge heparin-locked catheter (Master, Karachi, Pakistan) was inserted into the cephalic vein of the forearm for blood sample collection. Blood samples were drawn before the drug was given (zero time, baseline) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 and 24 h after the administration. Each time, a 5-ml blood sample was drawn after the first 0.5 ml was discarded for the removal of heparin in heparin-locked catheter. These samples were collected in labeled disposable plastic centrifuge tubes (Shifa, Karachi, Pakistan) containing 200 μ l of heparin. Blood samples were then centrifuged at 1,000 g, 24°C for 10 min. Then the upper plasma layer was removed and transferred into labeled sterile glass vials (5 ml). These plasma samples were immediately frozen at -20°C until assay. Blood sample analyses was repeated in the same manner in the second period to complete the crossover design.

Sample Preparation

The procedures described here were not only used for the extraction of study samples but also for the extraction of calibrators and quality controls. Plasma sample deproteination and liquid-liquid extraction procedures were as follows: 1 ml of plasma sample was transferred into a glass-stoppered tube (Shifa, Karachi, Pakistan) and 250 μ l of 20% sodium carbonate solution was added. The sample was mixed for 30 s using a vortex mixer. Then, 5 ml of cyclohexane was added and the samples vortexed for 3 min to achieve maximum extraction of drug to the organic phase. These samples were then centrifuged at 1,000 g for 10 min to achieve good separation of the two phases. The tubes were removed slowly and the upper organic layer was removed with great care using micropipettes and then transferred into another clean glass tube. These tubes containing organic phase were then placed in the chamber of the sample concentrator and the organic solvent was evaporated at 40°C under a gentle stream of nitrogen. When the sample was completely dried, the tubes were removed from the chamber. The dried samples were reconstituted in 100 μ l of the mobile phase and the reconstituted sample was vortexed for 30 s. The extracted sample was then transferred into a labeled Eppendorf tube and stored until injection into the LC system. The samples were extracted shortly before analysis.

A stock solution of nefopam was prepared by dissolving 100 mg of drug in 100 ml of methanol and was stored in a refrigerator (Dawlance, Pakistan). Standard curves were constructed to encompass the anticipated range of plasma nefopam concentrations found in fasting healthy subjects. Blank plasma was spiked with nefopam drug solutions to give concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 ng/ml. To achieve these concentrations in plasma, firstly a nefopam working standard solution of 6.4 μ g/ml concentration was prepared. This working standard solution was prepared by mixing 64 μ l of the stock solution in methanol to make a final volume of up to 10 ml. Then, 2 ml of blank plasma was pipetted into a centrifuge tube and 40 μ l of the above-mentioned working standard solution was added to give a final concentration of 128 ng/ml of nefopam. After mixing thoroughly us-

ing a vortex mixer for 30 s, 1 ml of this mixture was transferred to another labeled centrifuge tube and to this tube, 1 ml of blank plasma was added to make the final concentration of 64 ng/ml of nefopam. Similarly, other dilutions of 32, 16, 8, 4, 2, 1, 0.5 and 0.25 ng/ml were prepared by appropriate dilution with blank plasma.

The extraction procedure of study sample was the same as described above. 20 μ l of the extracted sample was injected into the HPLC system, mass spectra were recorded and peak areas were determined for each concentration. Intra-day (within-run) and inter-day (between-run) accuracies and precision of the assay were determined on 3 separate days.

Liquid Chromatography Mass Spectrometry Conditions and Method Development

Nefopam HCl (MW, 253.34 g/mol; purity 99.5%) was a gift from Mediceena Pharma, Lahore, Pakistan. All other chemicals were of analytical grade and were used without any alteration. Plasma nefopam was quantified based on a modified HPLC method that was validated before the study [2]. The HPLC conditions were optimized for the column (Eclipse XDB C₁₈; 5 μ m, 4.6 $\times$ 150 mm, column internal diameter 4.5 mm), mobile phase, and sample preparation. Analysis was performed by using high-performance liquid chromatography (Agilent 1100 series, Waldbronn, Germany) Mass Spectrometry, equipped with a pump (Agilent 1100 series) and a mass spectrometer (Agilent 1956 A VL) and LC/MS software ChemStation (Rev.A.10.02, Agilent Technologies). The mobile phase consisted of a 50:50 mixture of component A and component B. Component A was 1% formic acid in acetonitrile and component B was 1% aqueous solution of formic acid. The component A was prepared by mixing 5 ml of formic acid into acetonitrile to result in a final volume of 500 ml. Similarly, component B was prepared by mixing 5 ml of formic acid into double distilled water resulting in a final volume of 500 ml. Both of these components were mixed thoroughly in a large 1-liter measuring flask. Then the mobile phase was ultra filtered through a 0.45- μ m-membrane filter using a filtration assembly under vacuum. The filtered mobile phase was then degassed by placing the flask in an ultra sonic bath for 5 min. Then the mobile phase was transferred to the solvent bottle of the LC/MS for analysis. The mobile phase was freshly prepared every day. The mobile phase was pumped at a rate of 0.5 ml/min. The run time was 5 min.

Electrospray ionization parameters were as follows: positive mode, resolution normal, nebulizer pressure 35 psi, dry gas temperature 350°C and dry gas flow rate 9 l/min. Mass spectrum parameters for scan mode were: mass range 150–300, fragmentor 70, gain 1, threshold 150 and stepsize 0.1. Mass spectrum parameters for selective ion chromatogram mode (SIM) were: selected ion 254.1, fragmentor 70 and gain 1.

No significant interferences at the retention times of nefopam were observed on chromatogram of blank plasma under the aforementioned HPLC conditions. The analytical process was validated following the FDA guidance document for bioequivalence study [5]. The calibration curves were validated over a concentration range of 0.25–128 ng/ml for nefopam in human plasma with a lower limit of quantitation (LLOQ) of 0.25 ng/ml. Precision and accuracy were analyzed using four different concentrations [0.25 (LLOQ), 2, 8 and 32 ng/ml], and recovery was analyzed using three different concentrations (2, 8 and 32 ng/ml) of nefopam quality control sample.

Table 1. Comparison of mean $\pm$ SEM of bioavailability and pharmacokinetic parameters of Acupan and Inza administered in the oral dose of 30 mg

Series No.	Parameter	Acupan (3M) (mean $\pm$ SEM)	Inza (Mediceena) (mean $\pm$ SEM)	p value
1	C _{max} , ng/ml	60.74 $\pm$ 2.36	60.46 $\pm$ 1.29	0.867
2	T _{max} , h	1.63 $\pm$ 0.13	1.73 $\pm$ 0.07	0.096
3	AUC _{0-∞} , ng·h/ml	293.01 $\pm$ 16.39	307.43 $\pm$ 8.86	0.723
4	K _e , h ⁻¹	0.159 $\pm$ 0.005	0.146 $\pm$ 0.004	0.088
5	t _{1/2} , h	4.41 $\pm$ 0.16	4.80 $\pm$ 0.13	0.321

AUC = Area under the curve; K_e = elimination rate constant.

Tolerability

Health assessment including vital signs, physical examination and clinical laboratory testing of all subjects was performed before and 7 days after study. Subjects were interviewed at the beginning and end of each study period and were monitored throughout the study period to determine any adverse effects.

Pharmacokinetic and Statistical Analysis

Pharmacokinetic analysis was performed by using noncompartmental method of analysis [6] by using 'Thermo Kinetic' version 4.4.1 (Thermo Electron, San José, Calif., USA). Mean, SD and SEM were calculated using Microsoft Excel (Microsoft, Redding, Wash., USA). One way analysis of variance employing Statistical Package for Social Sciences (SPSS version 13.0) was used for testing for potential differences [7] between the pharmacokinetic parameters of the reference and test formulations, Acupan® and Inza®. The level of significant difference was set at $p > 0.05$. The following parameters were compared for bioequivalence testing: AUC_{0- ∞} and C_{max}. These parameters were log transformed prior to analysis and compared between treatments using one-way analysis of variance (90% CI). Equivalence was assumed if the 90% CIs of the test/reference ratios fell within the 0.80–1.25 acceptance limits.

Results

A representative ion chromatogram after injection of nefopam test solution is shown in figure 1. The ion chromatograms showed a retention time of nefopam at 2.3 min (fig. 2).

Pharmacokinetic Characteristics

Under the described analytical conditions, the relationship between the concentration and peak area ratio was linear from 0.25 to 128 ng/ml (LLOQ, 0.25 ng/ml) ($y = 54,472x + 2,661.4$; $r^2 = 0.9993$, x = plasma concentration; y = peak area). The intra-day accuracy of the method for nefopam ranged from 100.2 to 109.3%, while the intra-day precision ranged from 2.4 to 8.3%. The interday

Fig. 1. Mass spectrum of nefopam in scan mode.

Fig. 2. Chromatogram of a spiked plasma sample obtained in the SIM mode.

accuracy of this method was in the range of 99.8–102.4%, while the interday precision ranged from 3.8 to 9.7%. The recoveries were 21.2, 23.9 and 25.1% at 2, 8 and 32 ng/ml, respectively. The plasma drug concentration (mean $\pm$ SD) versus time profiles of the reference and test formulations after administration of 30-mg single doses are presented in (fig. 3). The mean $\pm$ SEM value of $C_{\max}$ ($\mu\text{g/ml}$) and $\text{AUC}_{0-\infty}$ ($\mu\text{g}\cdot\text{h/ml}$) for reference formulation was found to be 60.74 ± 2.36 and 293.01 ± 16.39 and for test formulation the values were 60.46 ± 1.29 and 307.43 ± 8.87 , respectively (table 1). The $C_{\max}$ and $\text{AUC}_{0-\infty}$ values of the test and reference formulations were nonsignifi-

cantly ($p > 0.05$) different. Also, there was no statistical difference of the $t_{\max}$ for reference (1.63 ± 0.13) and test formulation (1.73 ± 0.07). The half-life (h) and elimination rate constant (h^{-1}) for reference formulation was 4.41 ± 0.16 and 0.159 ± 0.005 while for test formulation 4.80 ± 0.13 and 0.146 ± 0.004 , respectively. Again, no significant differences were found between the two formulations for these parameters.

Bioequivalence

In the bioequivalence analysis, the 90% CIs obtained for the pharmacokinetic parameter test/reference ratios

Fig. 3. Comparison of log concentration versus time profile Acupan versus Inza plotted on a semi-log graph, administered in the oral dose of 30 mg in 12 fasting healthy Pakistani volunteers.

after log transformation were 1.0029–1.0146 and 0.9947–1.0033 with point estimation at 1.00875 and 0.9990 for $AUC_{0-\infty}$ and C_{max} , respectively. The differences between reference and test formulations were non-significant ($p < 0.05$) and the 90% CIs of the test/reference ratios of all relevant parameters fell within the bioequivalence acceptance limits of (0.80–1.25) as recommended by international bioequivalence regulatory guidance.

Tolerability

Nefopam was well tolerated by all of the subjects, with no clinically relevant adverse effects related to nefopam administration reported. Typical nefopam side effects include cough, dizziness, fatigue, somnolence, dyspepsia and vomiting, during or one week after the study.

Discussion

In this sample of fasting healthy Pakistani male young subjects, test and reference formulations of 30-mg nefopam tablets were found to be bioequivalent based on applicable regulatory guidance [5]. The assay had good sensitivity and used a simple liquid-liquid extraction with sodium carbonate solution-cyclohexane. The validation showed that the assay was adequate for extraction of nefopam from human plasma and its reliable quantification. Our results were similar to the previous pharmacokinetic study of Aymard et al. [2] which reported the following mean pharmacokinetic parameters – C_{max} , $AUC_{0-\infty}$, T_{max} – of 48.5 ± 18.9 ng/ml, 377 ± 193 ng·h/ml, 1.73 ± 0.12 h, respectively. However, there was a little

difference which could be related to the aldosterone synthase, cytochrome P450 (CYP) 11B2 gene which is the key rate-controlling enzyme in the final steps of aldosterone biosynthesis in the zona glomerulosa cells of human adrenal glands, and its expression is primarily regulated by angiotensin II and serum potassium levels [8]. Significant variability in the types and frequencies of CYP allelic variants has been found among ethnic groups [9].

Limitations of the Study

The main limitation of this study is that only 12 male young subjects were enrolled; therefore, the findings may not represent the pharmacokinetic characteristics of the local population. Hence, there is a need to include fed subjects, females, and children.

Conclusion

The results showed that both formulations possessed almost the same relative bioavailability and pharmacokinetic parameters.

Acknowledgement

The authors would like to express their gratitude to Dr. Nadia Cheema, Tahira Perveen Fatima Rasool, Usman Ghani and Shujaat Ali Khan for help with pharmacokinetic calculations.

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