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Technological evaluation and equivalence assessment of lorazepam tablets in rabbits

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Four different oral lorazepam tablets (Tavor® tablets as reference preparation and three generic tablet formulations, A, B and C) were investigated after administration to 12 rabbits to evaluate their bioequivalence. A single 2 mg/kg dose was administered orally as powder and lorazepam plasma concentrations were determined by a validated HPLC method. Maximum plasma concentrations (C_{max}), of 207 ng/ml (reference), 198 ng/ml (A), 166 ng/ml (B) and 169 ng/ml (C) were achieved. Lorazepam appeared in the plasma at 0.66 h (T_{max}) for all formulations, probably because the disintegration step was bypassed due to the pulverization of the administered doses. Areas under the plasma concentration-time curves (AUC_{0-t} and $AUC_{0-\infty}$) were determined. The obtained AUC_{0-t} values were 556.57 ng h/ml (reference), 554.70 ng h/ml (A), 493.08 ng h/ml (B), and 487.88 ng h/ml (C). ANOVA results ($P \geq 0.05$) and 90% confidence intervals for the mean ratio (T/R) of AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} were within the EMEA acceptance range. Pharmacokinetic and statistical results of this study show that the four tested drug products (Tavor®, A, B, C) are to be considered bioequivalent and interchangeable in medical practice.

1. Introduction

In order for a generic drug product to be interchangeable with the pioneer (innovator or brand name) product, it must be both pharmaceutically equivalent and bioequivalent to it. According to the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA), pharmaceutical equivalents are drug products that contain identical active ingredients and are identical in strength or concentration, dosage form, and route of administration. Bioequivalence is a comparison of the bioavailability of two or more drug products. Thus, two products or formulations containing the same active ingredient are bioequivalent if their rates and extents of absorption are the same (EMEA 2001; Guidance for Ind. 1997; Porta et al. 2005).

The *in vitro* dissolution testing is performed as a relatively fast and inexpensive technique to evaluate pharmaceutical dosage forms before they are tested in clinical trials and represent a valuable predictor of the *in vivo* bioavailability of oral dosage forms (Itiola et al. 1996; Kressmann et al. 2002a). Dissolution tests can be used to assist in the optimization of a drug formulation, as a quality control tool, to assess the stability of the drug product, to assess the batch-to-batch quality of production, or to estimate similarity between drug products after certain changes, such as in the formulation, the manufacturing process and/or equipment, like in the case of generics (Guidance for Ind. 1997; Agrawal et al. 2004; Kressmann et al. 2002b).

The aim of this study was to evaluate, *in vitro* and *in vivo*, the physical-chemical equivalence and bioequivalence of lorazepam [7-chloro-5-(o-chlorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-one], LZM commercial tablets.

Tavor® is one of the most widespread registered medicinal products containing LZM. Generic drug products containing LZM have been registered in Italy by several pharmaceutical companies and are present in the Italian market since September 2001. Due to the wide medical prescription of products containing LZM, it seemed interesting, from a technological and biopharmaceutical point of view, to perform in vitro and in vivo studies to evaluate the rate of dissolution, uniformity of content, uniformity of weight and pharmacokinetic parameters (AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, T_{max}) on rabbits of a trade mark and generic tablet formulations containing LZM available in the Italian market.

2. Investigations, results and discussion

Dissolution tests can discriminate the formulation factors that may affect drug bioavailability. The US Pharmacopoeia 25 in the LZM tablets monograph stipulates that all tablets should have released into the dissolution medium an amount not less than 60% of the labelled amount of LZM at 30 min and not less than 80% after 60 min.

The dissolution tests were carried out on Tavor® 1-mg tablets and compared to the profiles obtained for the three generic products A, B, C. The dissolution profiles of products A, B and C with respect to Tavor® are shown in

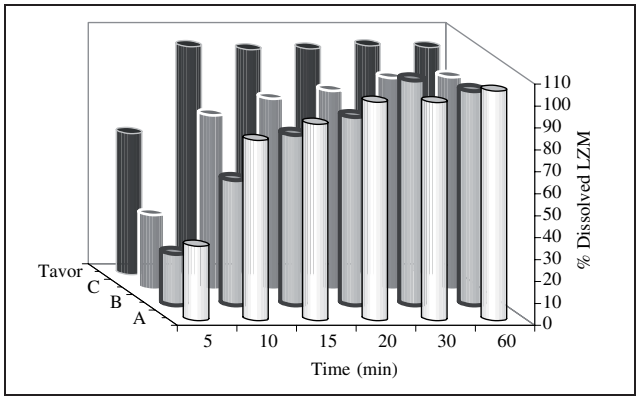

Fig. 1: Comparison of in vitro LZM dissolution profiles from Tavor®, A, B and C tablets

Fig. 1. Cumulative percentages of the drug dissolved from tablets were calculated and shown in Table 1. Each point represents the mean of twelve measurements.

All the products passed the dissolution test as reported in the US Pharmacopoeia 25; however, some differences were found between the dissolution profiles of the reference product (Tavor®) and the generic products.

Dissolution tests showed that the dissolution profiles were not parallel among the tested products. Only Tavor® ensured the availability of the whole labelled drug amount within 10 min. These differences may be due to the excipients used and/or the production process. Abdou et al. (1989), showed that the dissolution rate of a pure drug can be altered significantly when mixed with various excipients during the manufacturing process of solid dosage forms. For instance, Tavor® and B (1 mg) tablets have exactly the same qualitative and quantitative composition. The other two generic products show simpler formulations.

Therefore, the differences in drug dissolution observed for Tavor® and B tablets (Fig. 1 and Table 1) can be ascribed to a different and, in the latter case, less efficient production process.

The amount of LZM in the commercial tablets was determined as described in the European Pharmacopoeia (5th Edition). The obtained results are presented in Table 2. All products contained between 85% and 115% of the labelled amount of drug. There was no significant difference among the generic and reference products, even if product A showed the lowest content of active compound.

As regards the uniformity of weight all the tested products showed acceptable values, as none gave percent deviation in weight greater than 7.5%, as indicated by the European Pharmacopoeia (5th Edition) prescriptions. All the products were then within the tolerated limits. The percentage deviation of each tablet from the average weight was calculated (Table 3).

LZM-containing tablets (Tavor®, A, B and C) were administered to rabbits at a single dose of 2 mg/kg of active compound. The mean plasma levels of LZM following administration are shown in Fig. 2 and the pharmacokinetic parameters are reported in Table 4.

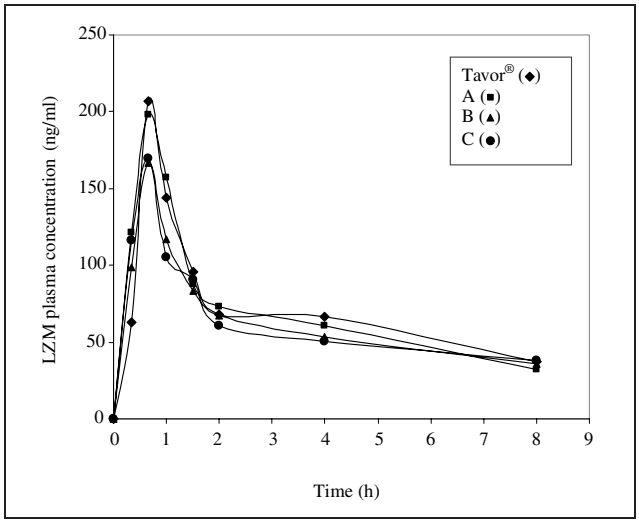

Fig. 2: Plasma concentration-time profiles of the four tested LZM tablets (Tavor®, A, B, C). Tablets were powdered and administered orally to rabbits at a drug dose of 2 mg/kg

Table 2: LZM content in the tested tablets (% of the labelled amount; n = 10)

Product	Mean drug content	S.D. (%)[a]	R.S.D. (%)[b]
Tavor®	100.8	0.7	0.7
A	91.5	1.2	1.3
B	96.8	1.9	1.9
C	99.9	1.6	1.6

[a] Standard deviation
[b] Relative standard deviation

Table 3: Results of the uniformity of weight test

Product	Mean weight (mg)	7.5% deviation	15% deviation
Tavor®	100.9	108.5	116.0
A	131.9	141.8	151.7
B	100.0	107.5	114.9
C	132.3	142.2	152.1

LZM appeared within 0.66 h (T_{max}) after the oral administration of all the four different compared formulations. This trend is probably due to the fact that all formulations were powdered before administration to rabbits so that it was bypassed the disintegration step, accelerating their dissolution rate. A different extent of LZM absorption (different AUC and C_{max} values) was observed for the four tested formulations. As shown in Table 4, the reference product exhibited the highest values of AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} followed by A, C and B products. In rabbits that received Tavor®, the higher LZM concentra-

Table 1: Mean time-correlation dissolution data for the four tested products [% of labelled amount of LZM ± S.D. (n = 12)]

Time (min)	Tavor	A	B	C
5	64.27 ± 0.90	33.92 ± 1.30	23.47 ± 1.00	34.10 ± 1.82
10	103.82 ± 1.01	82.17 ± 2.01	57.05 ± 1.04	79.57 ± 1.93
15	102.40 ± 1.82	89.55 ± 1.42	77.72 ± 1.03	87.12 ± 0.70
20	102.80 ± 1.10	99.60 ± 1.52	85.82 ± 0.94	90.75 ± 0.80
30	104.20 ± 1.10	99.42 ± 0.93	102.50 ± 0.82	96.52 ± 1.03
60	103.40 ± 0.92	104.65 ± 0.71	97.67 ± 0.92	97.00 ± 1.01

Table 4: Mean pharmacokinetic parameters (± SD) of LZM following administration of 2 mg/kg LZM in the four different oral formulations

Product	AUC_{0-t} (ng h/ml)	$AUC_{0-\infty}$ (ng h/ml)	t_{max} (h)	C_{max} (ng/ml)
Tavor®	556.57 ± 152.25	658.57 ± 91.11	0.66	207 ± 71.35
A	554.70 ± 84.27	631.23 ± 103.32	0.66	198.41 ± 54.95
B	493.08 ± 128.96	596.63 ± 70.42	0.66	166.42 ± 55.12
C	487.88 ± 140.74	615.48 ± 81.77	0.66	169.32 ± 68.16

Table 5: Statistical analysis of pharmacokinetic data

Pharmacokinetic parameters	ANOVA ($P \leq 0.05$)		
	Tavor® vs A	Tavor® vs B	Tavor® vs C
AUC_{0-t}	0.79	0.34	0.29
$AUC_{0-\infty}$	0.49	0.08	0.24
C_{max}	0.89	0.17	0.21
	(90% C.I.) Test vs reference ratio (% T/R)		
AUC_{0-t}	93.85–106.15	92.35–107.64	91.97–108.02
$AUC_{0-\infty}$	96.19–103.80	96.52–103.47	96.51–103.48
C_{max}	89.27–110.72	88.37–111.62	86.80–113.19

tion during the first 0.66 h could be due to its faster dissolution rate with respect to the other formulations. In fact, dissolution profiles (Table 1) showed that approximately 100% of LZM was released from Tavor® at 10 min, while only 82%, 57% and 79% was released at the same time point from formulations A, B and C, respectively. The higher values of AUC_{0-t}, $AUC_{0-\infty}$ may be ascribed to the higher mean amount of active compound contained in the reference drug product. As shown in Table 2 Tavor® had the highest LZM content.

EMEA established criteria to assess bioequivalence using statistical analysis on the obtained pharmacokinetic parameters (AUC_{0-t}, $AUC_{0-\infty}$, C_{max}) of different drug products (EMEA 2001). Bioequivalence is assessed by means of analysis of variance (ANOVA) and calculating standard 90% confidence intervals of the ratio test/reference (T/R) using logarithmic transformed data. The drug products are considered bioequivalent if the difference between two compared parameters is found statistically nonsignificant ($P \geq 0.05$) and 90% confidence intervals for these parameters falls within 80–125%.

The statistical comparison of AUC_{0-t}, $AUC_{0-\infty}$, C_{max} clearly demonstrated no significant difference in the four brands of LZM tablets. ANOVA results ($P \geq 0.05$) and 90% confidence intervals for the mean ratio (T/R) of AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} were within the EMEA acceptance range (Table 5).

Based on the pharmacokinetic and statistical results of this study, we can conclude that the four tested drug products (Tavor®, A, B, C) are to be considered bioequivalent and therefore interchangeable in medical practice.

Nevertheless, it is noteworthy that the percent ratio T/R of the C_{max} value for the generic product C (86.80–113.19) is close to the lowest limit of EMEA acceptance range, then the possibility exists that product C could not ensure the same therapeutic profile than Tavor®.

3. Experimental

3.1. Materials

This study was conducted using Tavor® tablets (1 mg) (Wyeth Medica Ireland) as reference product and three generics, named A, B and C, among the most diffused in the Italian market. All the tablet formulations were purchased from a chemist's shop. Standard LZM, used as a reference for all analytical methods, was obtained by Sigma Aldrich Chimica Srl (Milan, Italy). All other materials and solvents were of analytical reagent grade. The production details of LZM commercial products are given in Table 6.

Table 6: Properties of the tested LZM products

Product	Dosage form	Dose	Expiry Date	Country of origin
Tavor®	Tablets	1 mg	May, 2006	Italy
A	Tablets	1 mg	November, 2008	Italy
B	Tablets	1 mg	April, 2006	Italy
C	Tablets	1 mg	November, 2008	Italy

3.2. Dissolution tests

Dissolution studies were carried out on twelve tablets from each brand randomly selected using an ERWEKA DT apparatus as per USP <711> Apparatus 1 (basket), according to the USP monograph of Lorazepam Tablets (US Pharmacopoeia 25, First Supplement). The dissolution medium consisted of 500 ml of deionised water maintained at 37 ± 0.5 °C. The basket rotation speed was kept at 100 rpm. In all experiments, 1 ml of the dissolution sample was withdrawn at 5, 10, 15, 20, 30 and 60 min and replaced with fresh medium to maintain a constant volume. Samples were filtered (0.2 μm nylon membrane, Millipore) and assayed by HPLC, as reported below.

3.3. Assay of active ingredient

Assay of active ingredient was carried out as described in the European Pharmacopoeia (5th Edition) tablet's monograph. Tablets with a content of active substance less than 2 mg comply with test A for uniformity of content of single-dose preparations. Ten dosage units by each brand by random taken were transferred into a 50 ml volumetric flask. Twenty-five ml of methanol/water (85/15 v/v) mixture (diluent solution) were added. Each sample was sonicated for 10 min and magnetically stirred at 100 rpm for 20 min, then diluent solution was added to volume. The obtained suspension was stirred for 10 min and a portion of the suspension was then centrifuged for 10 min at 2000 rpm. A quantitatively measured volume of the clear supernatant was diluted with the diluent solution to obtain a test solution containing about 0.1 mg/ml of LZM. The obtained test solution was quantitatively compared to a standard solution obtained by dissolving an accurately weighed amount of standard LZM with the diluent solution to obtain a known concentration of about 0.1 mg/ml. Both test and standard solutions were analysed by HPLC. The amount of LZM in each tablet was calculated by Eq. (1):

$$100(C/10)(V_u/V)(r_u/r_s) \quad (1)$$

in which C is the concentration, in mg/ml, of LZM in the standard solution; V_u is the final volume of the test preparation; V is the volume, in ml, of the clear supernatant taken to prepare the test solution; r_u and r_s are the peak responses obtained from the test and the standard solution, respectively. The preparation complies with the test if each individual content is between 85% and 115% of the average content. The preparation fails to comply with the test if more than one individual content falls outside these limits or if one individual content is outside the limits of 75% to 125% of the average content.

3.4. Uniformity of weight

Uniformity of weight tests were carried out according to the European Pharmacopeia (5th edition). Twenty tablets randomly selected from each brand were weighed individually and their average weight was calculated. The percentage deviation of each tablet from the average weight was calculated. For tablets weighing between 80 and 250 mg the percent deviation of each tablet from the average weight is fixed at 7.5%. Not more than two units of the individual weights must deviate by more than the percentage deviation (7.5%) and none must deviate by more than twice that percentage.

3.5. Bioavailability studies

Bioequivalence studies were performed on a group of twelve New Zealand females White rabbits weighing approximately 3.5 ± 0.150 kg. The clinical protocol was approved by the local Ethics Committee. Animal room controls were set to maintain temperature at 19 ± 2 °C and relative humidity at $55 \pm 10\%$. There were approximately 15 to 20 air changes per hour and the rooms were lit by artificial light for 12 h each day. The animals were housed individually in stainless steel cages. Drinking water was supplied *ad libitum* to each cage by an automatic watering system. A commercially available laboratory rabbit diet was offered *ad libitum* throughout the study.

The study was performed according to a single oral dose Latin-square crossover design divided into four periods, each followed by two-weeks washout time. Tablets of all drug products (Tavor®, A, B, C) were crushed separately in a porcelain mortar to obtain a powder. The dosage amount (2 mg/kg of LZM) was suspended in a mixture of polysorbate 80, methylcellulose 4000 cps and distilled water (2 : 0.5 : 97.5 w/w). The suspension was administered by means of an intragastric rubber catheter (Nelaton CH 14 Fr) and an appropriate size syringe. After each dose, 2 ml of water were administered by the same method.

3.6. Sample collection

For LZM assay, approximately 2 ml of blood samples were taken from the ear vein and poured into sodium heparinized tubes prior to dosing and at 0.33, 0.66, 1, 1.5, 2, 4 and 8 h after dosing. The blood samples were centrifuged at 3000 rpm for 15 min at 4 °C. Plasma was separated and kept frozen at −70 °C until the analysis.

3.7. Sample analysis

To 1 ml of plasma, obtained as before described, 1 ml of a 2% (w/v) $ZnSO_4$ solution in water/methanol (70/30 v/v) was added to precipitate plasma proteins. Samples were vortexed for 5 min, stored at 4 °C for 10 min to precipitate proteins and then added with 4 ml of diethyl ether (McIntyre et al. 1993). The mixture was vortexed again and centrifuged at 3000 rpm at 4 °C for 15 min to allow separation of organic phase. After freezing of the aqueous phase, the organic upper layer was separated with a glass pipette and dried under reduced pressure. The obtained solid samples were solubilized in 400 µl of methanol, filtered through a 0.2 µm nylon syringe filter (Millipore) and analysed by HPLC for LZM concentration.

3.8. HPLC

The HPLC apparatus consisted of a Hewlett-Packard model 1050 liquid chromatograph (Milan, Italy), equipped with a 20 µl Rheodyne 7125 injection valve (Rheodyne, Cotati, Ca, USA) and an UV-VIS detector. The chromatographic analyses were performed on a Lichrosphere® 100 C_{18} RP column (particle size, 5 µm; 250 × 4 mm I.D.; Merck, Darmstadt, Germany), equipped with a 5 µm Lichrosphere® 100 C_{18} RP guard column (4 × 4 mm I.D.) (Merck) and eluted isocratically at room temperature with a mixture of methanol/$NH_4H_2PO_4$ buffer solution (0.05 M, pH 6.5) (63 : 37, v/v). Drug detection was carried out at a λ_{max} 230 nm and the flow rate was set at 1 ml/min. Calibration curve for quantitative evaluation of LZM was linear in the range 0.03–126 µg/ml ($r^2 = 0.9999$). The retention time of LZM was found to be 8.41 min.

For biological analysis, the calibration curve was obtained by spiking drug-free plasma with varying amounts of LZM (0.03–126 µg/ml) and treating the plasma samples as described above (see section 3.7.). A good linear relationship was observed between the concentration of LZM and the drug peak area with a high correlation coefficient ($r = 0.99983$) in the range of 0.04–126 µg/ml. Whenever a lower amount of drug less than the lower limit of HPLC quantification (30 ng/ml) was observed, the extraction procedure was repeated with larger plasma sample volumes. The HPLC method was validated for precision and accuracy. The drug-spiked plasma samples were prepared freshly on three different times of the same day and on three different days, and the resultant plasma samples were treated as described.

The intra-day and inter-day variations of the HPLC method were found to be less than 2.3% (CV) and less than 2.2% (CV), respectively, showing that this HPLC method was highly reproducible. When a known amount of LZM (100 ng) was added to preanalysed plasma samples (100, 200 or 400 ng/ml) and assayed using the present HPLC method, the observed concentrations (199.8, 297.8 or 499.2 ng/ml) were nearer to the expected concentrations (200, 300 or 500 ng/ml), evidencing that the method is highly accurate (mean accuracy 99.66%).

3.9. Pharmacokinetic analysis

The peak plasma LZM concentration (C_{max}) and the time to reach peak levels (T_{max}) were obtained from the plots of time vs plasma concentration of LZM. The area under the LZM concentration vs time curve to the last measurable concentration ($AUC_{0\rightarrow t}$) was calculated by means of the Origin Lab 7 software package. The AUC extended to infinity ($AUC_{0\rightarrow\infty}$), which represents the extent of bioavailability of a drug, was calculated using Eq. (2):

$$AUC_{0-\infty} = AUC_{0-t} + C/K_e \quad (2)$$

where C is the plasma concentration of LZM at the last time-point t and K_e is the apparent overall elimination rate constant calculated from the slope of the terminal elimination phase of a semi-logaritmic plot of concentration vs time, after subjecting it to linear regression analysis. Assuming the elimination to be a first-order process, K_e is represented as:

$$K_e = -\text{slope} \times 2.303 \quad (3)$$

3.10. Statistical analysis

For the purpose of bioequivalence analysis, AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} were considered as primary variables. Bioequivalence was assessed by means of an analysis of variance (ANOVA) and calculating standard 90% confidence intervals of the ratio test/reference (T/R) using logarithmic transformed data as recommended by EMEA (EMEA, 2001). The drug products were considered bioequivalent if the difference between two compared parameters was found statistically not significant ($P \geq 0.05$) and 90% confidence intervals for these parameters fell within 80–125%.

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