Jordan Journal of Pharmaceutical Sciences, Volume 3, No. 2, 2010

A Comparative Enzymatic Inhibition Assay as a Surrogate Indicator of Pharmaceutical and Potency Equivalence of Two Orlistat Formulations

Mohammad Mohammad[1]✉, Khaled M. Aiedeh[1], Hatim S. AlKhatib[1], Khaled Tawaha[1], Bashar AlKhalidi[1], Ihab M. Al-Masri[2], Saja Hamed[3] and Yasser Bustanji[1]

[1] Faculty of Pharmacy, University of Jordan, Amman, Jordan.
[2] Faculty of Pharmacy, Al-Azhar University, Gaza, Gaza Strip.
[3] Faculty of Allied Health Sciences, The Hashemite University, Zarqa, Jordan.

ABSTRACT

The most practical measure of therapeutic equivalence between two commercially available and generic formulation of a certain drug is to determine their *in vivo* bioavailability. However, for the oral dosage form that is not intended to be absorbed (e.g. orlistat), *in vivo* bioavailability studies are irrelevant to the achievement of the product's intended purposes. However, specific requirements for these drug products may be set in a way that they should meet acceptable in *vitro* standards. For this purpose, a comparative enzymatic inhibition assay of the target enzyme, pancreatic lipase, was developed to demonstrate orlistat products' pharmaceutical and potency equivalence. In this study we compared the pancreatic lipase inhibition that is achieved by two orlistat formulations; a generic product manufactured by local company (Jordan Sweden Medical Company, JOSWE) and the reference one Xenical® manufactured by Roche. The inhibition was expressed by the concentration of product which inhibits 50% of the activity of the pancreatic lipase enzyme (IC_{50}). The results of these studies showed that both formulations have equivalent potency that was demonstrated by *in vitro* studies.

Keywords: Orlistat, Therapeutic equivalence, Enzyme inhibition, Pancreatic lipase

INTRODUCTION

Obesity is a disease characterized by excess weight gain. It affects over 300 million people world-wide, and is associated with increased risk in morbidity and mortality [1-3]. Moreover, obesity is a major risk factor for cardiovascular disease, diabetes mellitus type 2, dyslipidaemia (increased low-density lipoprotein cholesterol and triglyceride levels, decreased high-density lipoprotein cholesterol levels), high blood pressure, coronary heart disease and gallbladder disease [3]. Weight gain of more than 10 kg carries a significant increase in risk of these diseases without necessarily reaching the epidemiological threshold for obesity. However, weight loss of 5–10% of the body weight is associated with substantial improvement in the health of obese patients and a reduction in the incidence of type 2 diabetes mellitus [1-4]. These facts clearly illustrate the need for treating individuals early in the disease process of excessive fat accumulation, before they are considered clinically obese.

Conventional, non-pharmacological interventions, based on diet and exercise have limited long-term success in achieving weight loss in obese patients. Therefore, a number of pharmacological agents have been used in combination with dietary intervention and/or lifestyle interventions to reduce body weight [5].

A major factor that contributes to the development and maintenance of obesity is the excessive intake of dietary triglycerides. Thus, inhibition of pancreatic lipase which leads to prevention of lipid absorption is an acceptable approach for treatment of obesity. The only clinically approved as pancreatic lipase inhibitor is

Received on 8/10/2009 and Accepted for Publication on 17/12/2009.
✉ E-mail: mkmohammad@ju.edu.jo

orlistat [6, 7].

Orlistat (Fig. 1), a highly lipophilic hydrogenated derivative of lipstatin, is a potent and selective inhibitor of pancreatic lipase which is a key enzyme in the digestion of dietary fat [8, 9]. Dietary fat (triglycerides) must be hydrolyzed by this enzyme, converting them to free fatty acids (FFA) to monomers and monoglycerides, and then incorporated into bile acid-phospholipid micelles before they can be absorbed by the small intestine and enter the peripheral circulation as chylomicrons. Orlistat irreversibly inhibits triglyceride hydrolysis, reducing the absorption of FFAs and monoglycerides by about one-third [8, 9]. The undigested dietary fat passes through the gastrointestinal tract and is excreted in the stools. The action of orlistat is useful in the treatment of obesity since it would inhibit intestinal fat absorption and eventually reduces fat deposition and deprives the body of ingested calories

Presently, orlistat is marketed by Roche under the trade name of Xenical or by GlaxoSmithKline as over-the-counter product under the name of Alli. It is indicated for obesity management including weight loss, weight maintenance and reduces the risk for weight regain [10]. Orlistat is generally well tolerated, with gastrointestinal adverse events being the most commonly reported side-effects [10]. Orlistat, in addition to lifestyle and dietary intervention, is thus an attractive option for the treatment of patients who are obese and who have associated co-morbidities or those who are at risk of developing diabetes mellitus type 2. Absorption of oral orlistat is minimal with no evidence of accumulation after long-term administration. Orlistat is rapidly eliminated and excreted primarily in the faeces [11].

The most practical measure of a drug product's performance is to determine the *in-vivo* bioavailability of the drug. For some well-characterized drug products and for certain drug products in which bioavailability is self-evident (e.g., sterile solutions for injection), *in vivo* bioavailability studies may be unnecessary or unimportant to the achievement of the product's intended purposes. However, there may be specific requirements for certain drug products that they should meet an acceptable *in vitro* standard. The FDA waives the requirement for submission of *in vivo* evidence demonstrating the bioavailability of the drug product if the product meets certain criteria (U.S. Code of Federal Regulations, 21 CFR 320.22) [12]. An example of these drug products is the oral dosage form of a drug that is not intended to be absorbed (e.g., an antacid or a radio-opaque medium). In this case bioavailability is not required but instead, specific *in vitro* bioequivalence studies may be required by the FDA. For example, the bioequivalence of cholestyramine resin is demonstrated *in vitro* by the binding of bile acids to the resin [12].

Similarly, for demonstration of orlistat bioequivalence, *in vitro* enzymatic inhibition of the target enzyme, pancreatic lipase, can be considered as a surrogate indicator of *in vivo* bioequivalence considering the fact that absorption and blood levels of orlistat are not relevant to its therapeutic use as the site of action is the intestinal lumen.

JOSWE has developed a generic formulation of orlistat which is expected to provide, in the gastrointestinal tract of subjects, similar *in vivo* delivery rate of drug as the innovator formulation. Since the bioavailability of orlistat is not an indicator for its pharmacological activity and the binding of orlistat to pancreatic lipase is essential for its action, *in vitro* pancreatic lipase inhibition was used as a pharmacological end point to ensure the equivalence between the generic capsule formulation and the innovator formulation (Xenical® 120mg) produced by Roche. The inhibition activity was expressed in terms of the concentration of product which inhibits 50% of the activity of the pancreatic lipase enzyme (IC_{50}).

MATERIALS AND METHODS

Materials:

Pnacreatic lipase type II, (Sigma, USA), P-nitophenol butyrate (Sigma, USA), NaCl (Sigma, USA), Tris base (molecular biology grade, Promega, USA) and Sodium taurocholate (Sigma, USA). Xenical® 120mg capsules were purchased from the market and Olistat120mg capsules manufactured and provided by JOSWE.

Preparation of Test (T) and Reference (R) working solutions:

Both test (JOSWE orlistat) and reference (Xenical®) products were kept in the same conditions and treated in the same way in all the steps of the experiment. Stock solution of test product was prepared by dispersion of the content of 6 capsules in 600 ml of buffered 0.1 % sodium taurocholate (Tris HCl buffer pH=7.4, with 2.5mM NaCl) with continuous stirring (using a magnetic stirrer with 400 rpm) for one hr. Then 5 ml of this solution was centrifuged for 10 min under 14000g and the supernatant was taken. This solution was considered as the stock solution for the test product and was used to prepare eight serial diluted solutions using the same buffer. Dilution factor was in a range of 5-640 to give a final theoretical orlistat concentration range of 1.9-240 µg/mL (see table 1). Reference product (Xenical®) was treated with the same procedure and other eight different solutions of Xenical® were prepared (see table 2). The effect of all prepared solutions of test and reference on pancreatic lipase activity was evaluated (see below).

Measurement of Pancreatic Lipase Activity

A commonly used procedure for investigating pancreatic lipase activity was adopted in this study. It makes use of p-nitrophenyl esters like p-nitrophenolbutyrate (PNPB)[13, 14]. The release of p-nitrophenol is measured spectrophotometrically at wave length of 410 nm. The assay procedures are described previously, briefly: the reaction mixture is prepared with 0.1 mL of pancreatic lipase (200 unit/ml in 2.5 mmol of tris-HCl buffer, pH 7.4 2.5 mmol NaCl) and 1 mmol of PNPB dissolved in 5 µL acetonitrile. The volume of inhibitor (test or reference) is fixed to 5 µL and the overall reaction mixture is completed into 1 ml using a tris -NaCl buffer.

The reaction is started by adding the substrate to the reaction mixture. The release of p-nitrophenol is measured as the increase in absorbance at 410nm in an ultraviolet-visible spectrophotometer against blank using denaturated enzyme.

The pancreatic lipase activity is related to the rate of p-nitrophenol release which can be estimated from the slope of the linear segment of absorbance *vs* time profiles. Therefore, the inhibitory activity can be calculated from this formula

$$\%\ \text{of inhibition} = (1 - \frac{Si}{S}) * 100$$

Where S is the slope of linear segment of absorbance *vs* time profile in the absence of the inhibitor (control) and Si is the slope of linear segment of absorbance *vs* time profile in the presence of inhibitor.

The IC_{50} of the test sample can be obtained from the least-squares regression line of the plots of the logarithm of the sample concentration versus the pancreatic lipase inhibition (%).

RESULTS

The measurement of capability of both products (test and reference) to inhibit the target enzyme, pancreatic lipase, *in vitro* was used as surrogate indicator of *in vitro* bioequivalence of the two orlistat formulations (generic and innovator). It is evident from table 1 and table 2, that the inhibitory effect of orlistat in the test or reference is dose dependent. Different solutions of Xenical and test product were prepared in escalating doses and tested against the lipase activity. Table 1 shows how the activities of lipase decreased by increasing the theoretical orlistat concentration, similar results are shown the table 2 regarding the orlistat of reference product (Xenical). Moreover, to estimate the degree of similarity between the effects of the two products both the values of IC_{50} for both products were determined and the relationship between the concentration and the degree of lipase inhibition was plotted in figure 2.

Estimation of IC_{50} For both Test and Reference products:

IC_{50} value is commonly used to express the potency of enzyme inhibitors and it represents the concentration of the inhibitor at which 50% of the enzyme is inhibited. IC_{50} values for both test and reference were estimated by studying the effect of various concentrations of both

products on the degree of the inhibition. Figure 2 portraits the effect of escalating concentrations of both the test and the reference on the pancreatic lipase activity. This figure clearly shows the similarity of the behavior between the enzymatic inhibition profiles for both products. It is evident that by increasing the concentration of the test or reference products the degree of enzyme inhibition is increased in logarithmic manner. However, the inhibition continues until reaching a plateau which indicates the maximum inhibitory capacity of the drug.

IC_{50} values can be estimated by least-square regression of the linear segment of theoretical concentration *vs* degree of inhibition curves. However, in the determination of the IC_{50} value, a linear segment of at least four points of each curve was considered in the analysis (figure 3 and 4). Two of the measurement points have shown enzymatic inhibition more than 50% and the others produced enzymatic inhibition less than 50%. Figure 3 and 4 show the inhibitory effect of JOSWE Orlistat and Xenical on pancreatic lipase, respectively. The results of regression and equations are also shown in the figures.

Using the results of the regressions, IC_{50} values were estimated for test and reference products to be 19.59 µg/mL and 19.80 µg/mL, respectively.

Statistical analysis (using DataFit 2 program) for these data have shown that there is no significant difference between the two regression equations of the test and reference products ($p > 0.1$) thus the IC_{50} values of test and reference are considered similar and this is obvious since the values are very close to each other.

Further analysis of the data were carried out by PLA 2.0 software package, this software is designed for analysis of biological or potency assays with the help of the parallel-line or parallel logistics methods [15]. Results have shown that the two curves (Test and Reference) are equivalent with potency ratio of 99.3 % and 90% confidence interval of 83.7-117.8.

DISCUSSION AND CONCLUSIONS:

Dissolution profiles of the test and reference products were compared by calculating the similarity factor (f2) [FDA guidance for industry, 1997] (data are not shown). Generally f2 values should be greater than 50 to ensure similarity of the two dissolution profiles. However, the similarity factor (f2=71.4) between the *in vitro* dissolution profiles of the generic and innovator formulations ensured the equivalence between them.

The capsules of orlistat contain ingredients other than the active ingredient itself, orlistat. The presence of these ingredients in the capsule could have a positive, neutral or negative effect on the inhibitory action of orlistat on the pancreatic lipase. To investigate the possible effects of such ingredients on the pancreatic lipase or the effect of these ingredients on orlistat itself, an *in vitro* enzyme inhibition assay was suggested.

The use of sodium taurocholate, a bile acid normally found in the intestine, in these experiments was intended to provide a close simulation of the solution conditions in the gastrointestinal tract. The USP recommends the use of sodium taurocholate as a component in biorelevant gastrointestinal media to be used for the dissolution testing of drugs. Sodium taurocholate presence in these experiments supports the credibility and reality of the *in vitro* study compared to the *in vivo* situation.

The FDA evaluation of bioequivalence of *in vivo* absorbed test products depend on the ratio of the mean value of the test product to that of the reference one with a confidence interval of (80 – 125). By analogy to these regulations, and according to the above results we can conclude that the two orlistat products are similar in terms of their enzyme inhibition activity, and can be considered bioequivalent based on the conducted *in vitro* performance testing.

ACKNOWLEDGMENTS

This project was sponsored by the Deanship of Scientific Research at the University of Jordan (Grant no. 905). The authors wish to thank the Deanship of Scientific Research for their generous funds. The authors would like to thank also Jordan Sweden Medical Company (JOSWE) for the donation of their generic product.

Table 1: Test product (JOSWE Orlistat) results

Theoretical Orlistat Concentration (µg/mL)	Degree of inhibition (%)
0 (without Inhibitor)	0.00
240	91.82
120	87.55
60	79.29
30	61.71
15	38.99
7.5	28.11
3.75	17.75
1.875	14.71

Table 2: Reference product (Xenical) results

Theoretical Orlistat Concentration (µg/mL) of Xenical	Degree of inhibition (%)
0 (without Inhibitor)	0.00
240	90.51
120	82.59
60	78.85
30	59.44
15	47.26
7.5	21.76
3.75	18.02
1.875	9.66

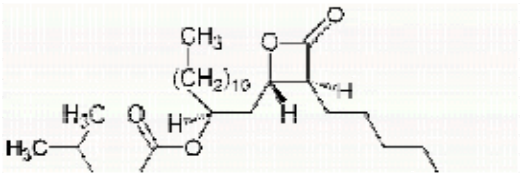

Figure 1: Orlistat structure

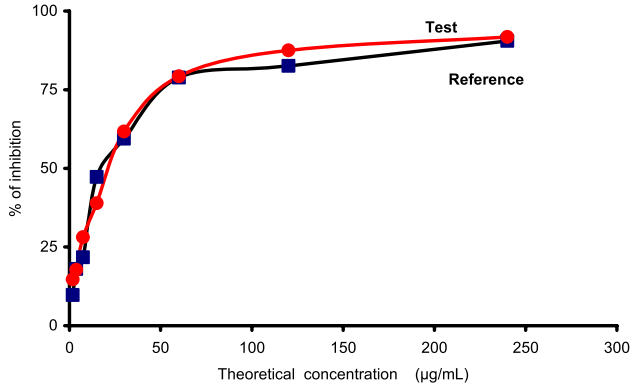

Figure 2: The pancreatic lipase inhibitory effect of both the test (●, red) and the reference (■, black) products.