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Direct Measurement of Lipase Inhibition by Orlistat Using a Dissolution Linked *In Vitro* Assay

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Abstract

Purpose: To develop a bio-assay that would be able to directly test gastrointestinal and/or dissolution samples to determine lipase activity and inhibition by Orlistat.

Methods: Enzyme assays were performed with porcine pancreatic lipase and para-Nitrophenyl Palmitate (pNPP) in pH 8.0 reaction buffer at 37°C. Substrate hydrolysis was monitored by absorbance changes at 410 nm. The dissolution of two Orlistat formulations was tested with a USP II apparatus. Samples were HPLC analyzed to determine release profile in addition to being diluted and directly assayed for inhibitory effect.

Results: The lipase-pNPP system demonstrates linearity and Michalis-Menten kinetics with a $K_m = 2.7 \pm 0.2$ μM and $K_{cat} = 0.019$ s^{-1}. Orlistat showed highly potent and time dependent inhibition with 5 ng/ml effecting 50% activity after 5 minutes in the Lipase-pNPP system. Dissolution studies showed a correlation of the drug release profile to the inhibitory effect of dissolution samples in the assay.

Conclusions: The lipase-pNPP method can be used as an *in vitro* assay to monitor orlistat inhibition from drug release or dissolution samples.

Keywords: Orlistat; Bioassay; Lipase; Inhibition; Dissolution

Abbreviations: pNPP: para-Nitrophenyl Palmitate; GI: Gastrointestinal; pNPA: para-Nitrophenyl Acetate; DPG: 1,2-Dioleoyl-3-Pyrenedecanoyl-rac-Glycerol; THL: Tetrahydrolipistatin; IVIVC: *In vitro-in vivo* Correlation

Introduction

Lipase plays a key role in dietary fat absorption by hydrolyzing triglycerides to monoglycerides and free fatty acids. As such, lipase inhibition may be a possible pathway to reduce total caloric intake. Orlistat is a potent lipase inhibitor that is sold as a weight loss aid in prescription and over-the-counter formulations. It has been demonstrated that orlistat is an irreversible inhibitor that covalently binds to serine 152 residue of lipase [1], which causes a pronounced *in vitro* as well as *in vivo* inhibition of gastric and pancreatic lipases [2,3]. Previous studies on this time dependent inhibition have used two-phase emulsion monitoring activity with a pH stat, which limits sample throughput [4,5].

Several studies have also revealed the significant effect of modifying the formulation of the orlistat on lipase inhibitory activity [6]. It was found in one study that nano-sized particles of orlistat can be used for enhanced *in vitro* dissolution rate and lipase inhibition [7]. However, to optimize the formulation for pharmacodynamic effect would require different time-consuming and costly clinical studies. Hence, there is a need for a simplified *in vitro* method to guide the selection of a suitable composition of formulation to optimize the release profile for obtaining better pharmacodynamic effect of orlistat products.

To address this need, a dynamic *in vitro* single-phase system was developed to monitor lipase inhibition, enabling rapid screening of multiple test samples. This system was able to provide a simulation of the in vivo lipase activity and is capable of directly testing dissolution or gastrointestinal (GI) fluid samples to assess lipase inhibition by orlistat. To validate the developed assay, two different formulations of orlistat were monitored for their lipase inihibitory activity from drug release samples.

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Materials and Methods

Materials

Porcine Pancreatic Lipase (Type II) (EC 3.1.1.3), sodium deoxycholate, sodium phosphate monobasic, isopropanol and p-nitrophenyl palmitate (pNPP) were purchased from Sigma Aldrich (USA). Tetrahydrolipistatin (Orlistat, THL) was provided by Roche. UV-transparent 96-well plates were used for colorimetric quantification on a Tecan Infinite M200 spectrophotometer.

Lipase preparation

Since the majority of triglyceride hydrolysis is done by pancreatic lipase, porcine pancreatic lipase was used as a model enzyme. Crude lipase was dissolved in reaction buffer (10 mg/ml) and centrifuged at 7000 g for 10 minutes to remove insolubles [8].

In vitro assay for determination of lipase inhibition

Lipase assays were performed in a 96-well, clear, flat bottomed plate with 200 μl reaction volume. pNPP was used as a substrate with a reaction buffer of 50 mM sodium phosphate, 5 mM sodium deoxycholate, and 10% isopropanol at pH 8.0 [9-11]. Lipase assays used a 200 μl reaction volume and substrate conversion was monitored with a Tecan Infinite M200 spectrophotometer at 410 nm. All assays were

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run at 37°C and reported results are the average of six replicates that were blank subtracted.

Estimation of enzyme kinetics

To characterize the system, the kinetics of this single phase system was analyzed. Enzyme linearity of was shown by finding the initial rate for increasing enzyme concentrations. For determination of the kinetic constants, enzyme concentration was fixed at 0.01 mg/ml (the lipase concentration was determined per an established calibration curve, data not shown) and the substrate concentration was varied. Minimization of the sum of squares gave a fit to the Michalis-Menten equation.

Estimation of time dependent inhibition

Pre-incubation and co-treatment studies were performed to verify single phase irreversible binding. For pre-incubation, the lipase and orlistat were added together and incubated at 37°C for varying times before substrate was added and the initial rate (<2 minutes) was recorded.

For co-treatment, the substrate and orlistat were added together and the rate was monitored continuously. To find the activity as a function of time, the derivative of a 2nd order polynomial fit was taken to find the instantaneous slope, which was normalized to the positive control.

Dissolution studies

Two solid dosage forms of orlistat were compared using this pNPP assay, Formulation 1 and Formulation 2. A Hanson USP II dissolution apparatus was used to determine release profiles of the formulations. The dissolution media used was 3% Sodium Lauryl Sulphate (SLS), pH 6.0 at 37°C. 2ml samples were manually withdrawn and filtered with a 0.45 μm Poly tetra fluoro ethylene (PTFE) filter, discarding the first ml of filtrate. Samples were analyzed on an Agilent 1100 HPLC with a Waters nova-pak C18 3.9×150 mm, 4 μ particle size HPLC column to determine the active concentration. Dissolution samples were diluted and incubated with lipase and tested for activity with pNPP.

Results

Enzyme kinetics

The enzyme demonstrated a high degree of linearity for the range of concentrations considered (0-2.5 mg/ml) as shown in figure 1. Lipase-pNPP single phase catalysis exhibits Michalis-Menten kinetics with a $K_m = 2.7 \pm 0.2$ μM and $K_{cat} = 0.019$ s^{-1} as shown in figure 2.
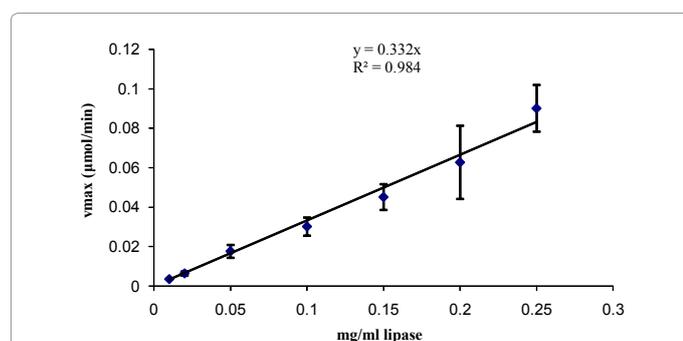
Figure 1: Lipase activity demonstrating linearity with pNPP (0.5 mM) at 37°C in reaction buffer (50 mM sodium phosphate, 5 mM sodium deoxycholate, 10% isopropanol at pH 8.0).
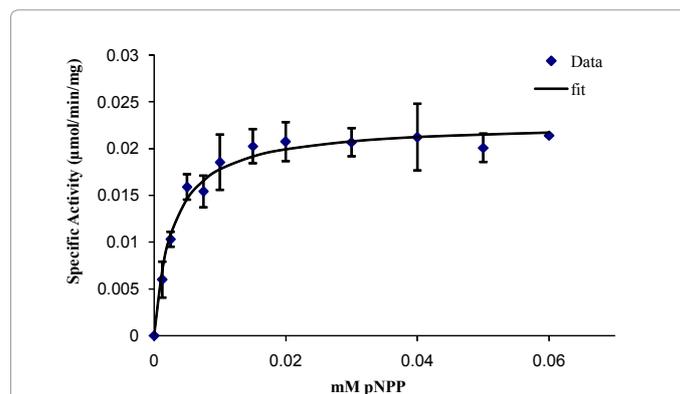
Figure 2: Lipase activity demonstrating Michalis-Menten kinetics ($K_m = 2.7 \pm 0.2$ μM and $K_{cat} = 0.019$ s-1) with pNPP at 37°C in reaction buffer (50 mM sodium phosphate, 5 mM sodium deoxycholate, 10% isopropanol at pH 8.0).
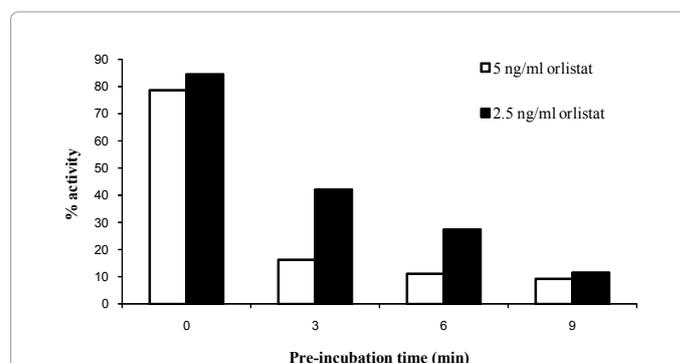
Figure 3: Pre-Incubation Inhibition Kinetics. 0.05 mg/ml lipase, 0.2 mM pNPP, 10% isopropanol, 50 mM Na phosphate pH 8, 5 mM Na deoxycholate.

Lipase inhibition

Lipase inhibition assays showed that orlistat is a potent, time dependent inhibitor in single phase solutions. In the pre-incubation study, an exponential decrease in the observed initial rate of the enzyme was observed as shown in figure 3.

Co-treatment inhibition kinetics showed a linear decrease in the enzyme activity with time (Figure 4). For the incubation results, all 2nd order fits had a very good fit ($R^2>0.99$), which provided linear decreases in the activity with time. Lipase activity was completely negated with 20 ng/ml orlistat in a sample with 0.1 mg/ml lipase.
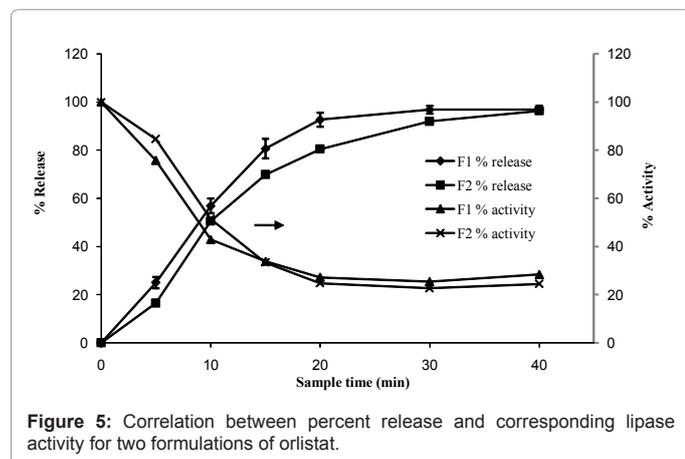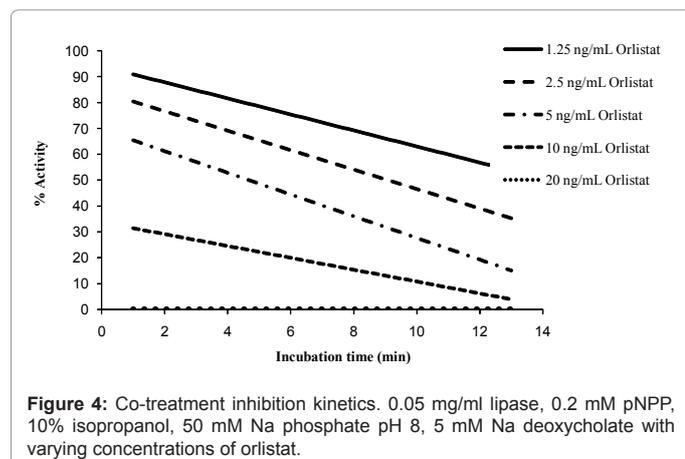
Dissolution studies

Typical and repeatable dissolution profiles were observed using a USP II apparatus. Two different formulations were able to be distinguished in the dissolution test under identical conditions, with Formulation 1 showing a faster initial release rate.

Dissolution samples produced an inhibitory effect that correlates to the release profile. The effects were most notable with the formulation 2, where small differences in the initial rate of release correspond to significantly higher inhibition (Figure 5).

Discussion

The study demonstrated the utility of an *in vitro* single phase assay that would be able to directly test GI and/or dissolution samples to determine lipase activity and inhibition by orlistat.

Figure 4: Co-treatment inhibition kinetics. 0.05 mg/ml lipase, 0.2 mM pNPP, 10% isopropanol, 50 mM Na phosphate pH 8, 5 mM Na deoxycholate with varying concentrations of orlistat.

Figure 5: Correlation between percent release and corresponding lipase activity for two formulations of orlistat.

The results confirmed and expanded the findings of previous two-phase studies that orlistat is an irreversible, time-dependent inhibitor of lipase [1,3,5,12-14], despite using a single-phase reaction.

In two phase emulsion studies, coalescence of emulsion particles may gave rise to reduced surface area to the point where reaction rates may become limited in part by substrate. In addition, the resulting heterogeneity caused by "creaming" of the emulsion may compromise the ability to procure representative subsamples for conducting the lipase assay. Therefore, the developed single phase assay is arguably better method to evaluate lipase activity [15].

Enzyme linearity over a range of physiologically relevant concentrations and adherence to Michalis-Menten kinetics provide a simple and robust system for testing dissolution or GI samples. Additionally, the resolution that this assay provides can distinguish between minor differences in enzyme inhibition. The ability to directly evaluate dissolution samples is validated by the correlation with the HPLC release profile. In combination with bio-dissolution system, the method could be developed as a bio-method to determine the suitability of formulation (such IR or SR) for orlistat-type drugs.

Limitations of this method may arise when examining interfacial effects of excipients. A potential example of this is sucrose ester, which alters the surface tension of micelles in an emulsion [16]. As such, its effects would not be able to be seen in a single phase assay.

Optimizing excipient formulations may allow for lower dosages of the active, but will require improved *in vitro-in vivo* correlations

(IVIVC) to predict pharmacodynamic behavior before human trials. Future studies using this assay with a biorelevant dissolution method and human lipase or GI fluid will verify the sensitivity of the assay in addition to providing a more accurate IVIVC.

Conclusions

We have developed a very sensitive, single-phase assay for orlistat that mimics the time-dependent inhibition seen in emulsions. Additionally, we have shown a successful proof of concept for using dissolution samples in an *in vitro* assay as a method of estimating *in vivo* efficacy.

Acknowledgements

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