ORAL LIPID LOADING TEST IN WISTAR RATS AS A RELIABLE METHOD FOR THE STUDY OF THE TRIACYLGLYCEROL MALABSORPTION

Prueba oral de carga de lípidos en ratas Wistars como procedimiento fiable para el estudio de la mala absorción de triglicéridos.

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ABSTRACT

Most of the lipid digestion is carried out by pancreatic lipase, secreted in the upper part of the intestinal lumen by pancreatic juice secretions. In this paper, we describe a new single stressless and fast method to evaluate plasma triacylglycerol levels on Wistar rats under anaesthesia and with minimal personal training. After the administration of different diet treatments, plasma triacylglycerol (TAG) levels were analyzed at 30, 60, 120, 180 minutes to characterize the lipidic gastrointestinal absorption. Orlistat, a known antiobesity drug, was used as positive control. The results shown that the method was able to differentiate the treatments administered utilizing up to five animals at each time step. In addition, with this method was shown that Orlistat treatment prevents TAG systemic uptake at 30 and 60 minutes compared to a control diet without it. On the light of these results we conclude that the method described in this study could be employed to evaluate short-term lipid digestion.

Keywords: Pancreatic Lipase, Digestion, triacylglycerol.

RESUMEN

La mayor parte de la digestión de lípidos se lleva a cabo por la lipasa pancreática, siempre en la parte superior del lumen intestinal por secreciones del jugo pancreático. En este artículo, se describe un nuevo método rápido y menos estresante para evaluar la actividad de la lipasa pancreática que se puede realizar por un solo operador. Con el fin de hacerlo, el plasma sanguíneo se analizó para caracterizar la cinética de absorción

gastrointestinal lipídica con diferentes tensoactivos fisiológicos y con el inhibidor de la lipasa pancreática Orlistat como control positivo. El resultado mostró que fue posible evaluar cinco animales en menos de 30 minutos con un operador y que el tratamiento con Orlistat consiguió prevenir la absorción sistémica de los TAG hasta 120 minutos después del tratamiento. A la luz de estos resultados se concluye que el método descrito en este estudio podría ser empleado para investigar el proceso de digestión de lípidos en diferentes condiciones.

Palabras clave: Lipasa pancreática, Orlistat, Digestión, Triglicéridos.

INTRODUCTION

The digestive process and assimilation from the small intestine play a significant role in the amount of calories that are ultimately supplied to the body. Most of the lipid digestion is carried out by pancreatic lipase, secreted in the duodenum by pancreatic juice secretions. The intestinal uptake of dietary fats is facilitated by the bile acids and phospholipids that act as lipid solubilizer in the oil/water interface within the gut lumen (Kleinsorgen et al., 1981; Staels et al., 2009). Lipid hydrolysis is heavily dependent on the action of pancreatic lipase and the oil/water interface. The enzyme works splitting fatty acids from their glycerol base in order to transport the individual components through the intestinal wall (Di Maio et al., 2010). The interface allows the enzyme to exert its function between the aqueous medium and insoluble lipid droplets (Reis et al., 2008). It results that inactivation of pancreatic lipase activity or the change in the interface composition, triacylglycerol (TAG) hydrolysis would be affected, resulting in a lesser blood circulating TAGs. Fewer methods *in vivo* have been described throughout the bibliography in order to evaluate TAGs blood level after a diet administration. These methods suffer of several drawbacks, including being invasive and needing skilled personal, or being too stressful for the animal and time consuming (Carrière et al., 1997; Eddouks et al., 2005; Uchiyama et al., 2011). In addition, these studies lack of standardization process which resulted in poorly described methods. In this paper, we describe a new single stress less and fast method to evaluate TAG levels that can be performed with one researcher. In order to do so, plasmatic TAGs were analyzed on Wistar rats to characterize the lipidic gastrointestinal absorption levels of different diets and with the pancreatic lipase inhibitor Orlistat as positive control.

MATERIALS AND METHODS

Animals

Thirty Male Wistar rats (7-8 weeks old) were obtained from University of Murcia Laboratory Animal Service. The rats were housed under a 12h light/12h dark cycle in a temperature and humidity-controlled room. Rats were given food and water *ad libitum* while water was withheld during the test. All procedures involving animals were approved by the Murcia Ethics committee.

Material

Virgin olive oil was obtained from a local market and was employed without further purification (Hacendado, Spain), Cholic acid, egg yolk lecithin (Sigma-Aldrich, St.Louis, USA), Orlistat (Allí, Spain), Capillary tubes (CB 300-microvette, Alemania), Isofluorane (Baxter).

Methodology

Prior to the experiment, rats were fasted for sixteen hours. In order to evaluate the best postprandial TAG systemic level curve for the Orlistat test, five rats were orally administered with 10 ml/kg via intragastric tube and treated with the following diets: olive oil 100 % (w/v)

[1], olive oil/distilled water 50:50% (w/v) with 0.5 % cholic acid [2], olive oil/distilled water 50:50% (w/v) with 1% cholic acid (w/v) [3], olive oil/distilled water 50:50% (w/v) with 1% cholic acid and 3 % lecithin (w/v) [4], olive oil/distilled water 50:50% (w/v) with 3 % lecithin (w/v) [5]. All the diets were compared with a group that received distilled water [Control]. Before the administration all the diets but the diet [1] were rapidly homogenized at 8000-10000 rpm with an Ultraturrax Homogeneizator (Ultraturrax T20, Denmark). Blood samples were collected by tail vein incision at 0, 30, 60, 120, 180 minutes after the oral administration under isofluorane anaesthesia (3% w/v) that was used only during the blood collection procedure. Blood was centrifuged for plasma and TAGs levels were measured with a commercial colorimetric assay (Olympus triglyceride OSR6133) in an Olympus AU600 autoanalyzer (*Olympus*, Japan). The technique is based in a series of combined enzymatic reactions. The final reaction permits the formation of a product with a maximum absorbance at 500 nm. Finally the TAGs in the sample were measured proportionally with the rise of the absorbance till 520/600 nm. Plasma TAGs and were expressed as increments from the baselines. Incremental areas under the response curves (AUC) during the whole time were calculated using the trapezoidal rule with fasting levels as the baseline. The diet which shown the best TAG absorption curve and AUC respect to untreated animals was employed as vehicle for Orlistat test treatment diet, added at a concentration of 60 mg/animal.

Statistical analysis

The values obtained were expressed as the mean±SD (standard deviation). Data followed a normal distribution as assessed by a D'Agostino-Pearson test, so parametric tests were employed to analyze the results. Differences of means among each time step and AUC treatments were analyzed with one-way ANOVA. Group differences were compared with Dunnett's post test. Statistical significance was defined as $p<0.05$. The analyses were carried out with Graphpad statistical software.

RESULTS

Dealing with the method, it was possible to manipulate five animals in each time step with one researcher. Sixteen hours fasting were necessary to stabilize TAG levels at the time of the treatment (data not shown). Each animal was anesthetized less than 5 minutes and was completely conscious for the next blood collection time step. In the control diet, the plasma TAG curve did not increase considerably from the baseline. In contrast, the other diets shared a common pattern, with a sharp increment at 30 minutes, reaching a maximum peak at 120 minutes and then decreasing to TAG baseline value at 180 minutes (Fig 1). Dunnett's Multiple Comparison Test reveals that the diet [5] promoted a significant TAG increment at 30 minutes ($p<0.05$), 60 and 120 minutes ($p<0.001$). At 120 minutes its value is ten times higher that the control. The diet [2] only had a significant increment at 60 minutes ($p<0.05$). The other diet treatments did not result in a statistically significant TAG increment at the time steps considered in the study. AUC results shown that only TAG levels diet [5] and [2] were significantly different versus the control ($p<0.001$ and $p<0.05$ respectively; Fig 2). Based on these results, diet treatment [5] was selected as vehicle control for the Orlistat test. In Fig. 3, Orlistat prevented TAG systemic uptake at 30 and 60 minutes ($p<0.05$) reaching values similar to the control. At 180 minutes the TAG absorption increased its value, reaching higher levels than those obtained with diet [5] alone at 180 minutes. AUC results (data not shown) shown that Orlistat, although did not have a statistically significant effect, decreased fat absorption of a one fifth compared to diet [5] treatment.

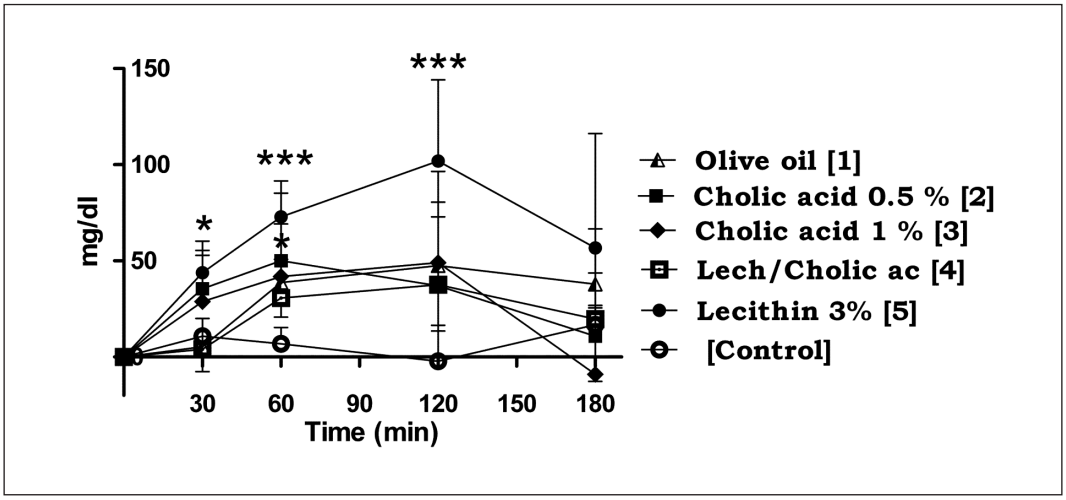

Figure 1. Plasma TAG values of the different treatments versus time of the experiment. All the diet treatments were compared to the control and data were analyzed with one-way ANOVA. The results were expressed as mean±standard deviation *p<0.05, *** p<0.001 n=5.

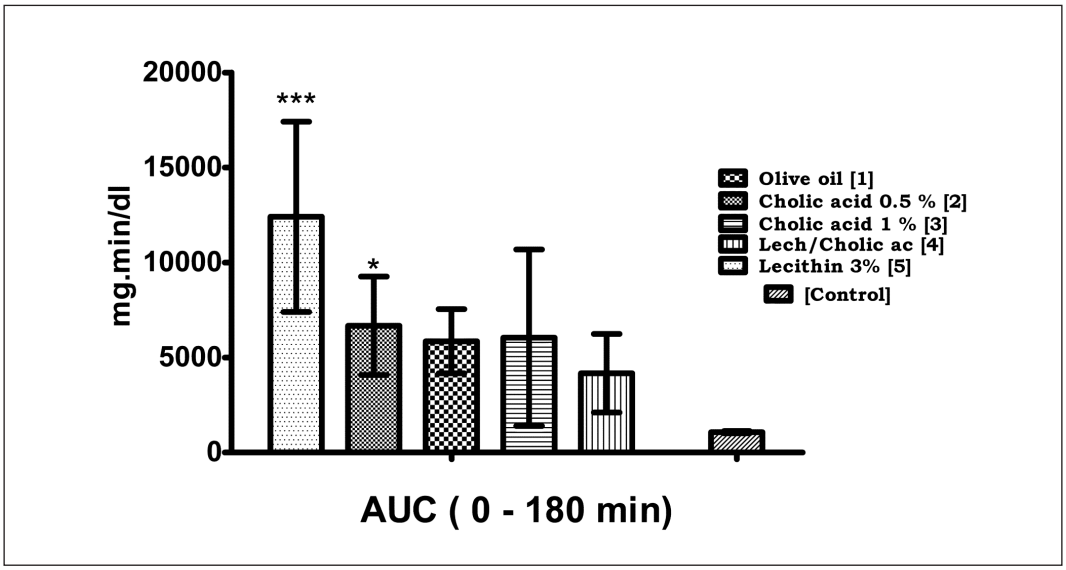

Figure 2. AUC during the three hours of the experiment. The data of the different diet treatments were analyzed with one-way ANOVA compared to the control. The results are expressed as mean ± standard deviation *p<0.05, *** p<0.001 n=5.

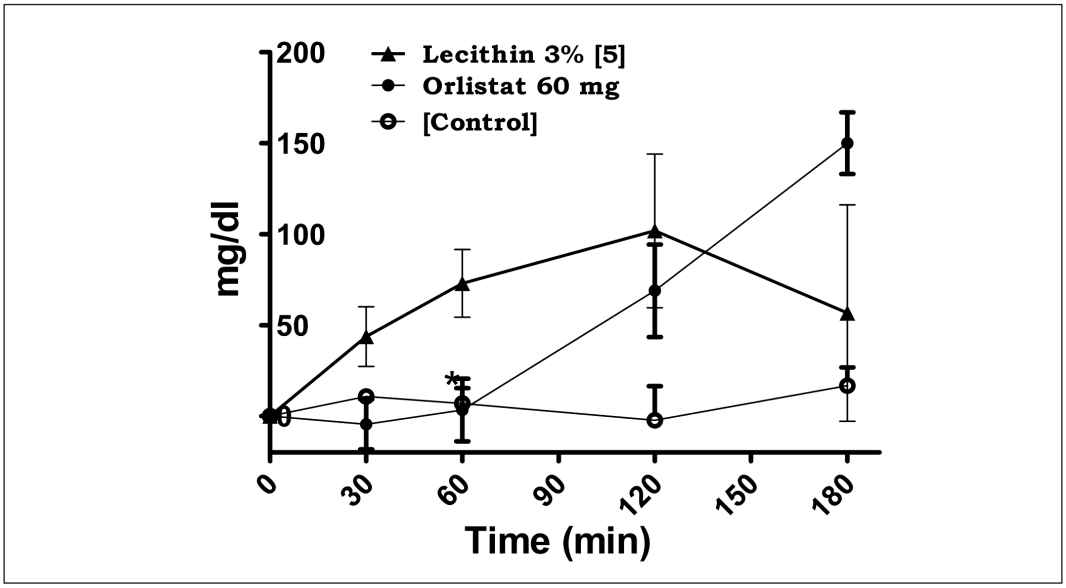

Figure 3. TAG blood levels between the lecithin 3% treatment and the same plus Orlistat. The data from the control were also included. The results were expressed as mean ± standard deviation *p<0.05 n=5. Statistically different values between lecithin 5% and Orlistat were shown at 60 minutes (p<0.05).

DISCUSSION

In this study we developed a simple and fast method to assess the pancreatic lipase activity during the lipid digestion. This procedure was carried in three hours. This time was enough to demonstrate different lipid treatment effects on the systemic TAG uptake levels in rats. In this method we did not fast the rats overnight which were likely to produce false baseline TAG values as reported elsewhere (Waner et al., 1994). We confirm that sixteen hours were necessary to keep a steady state baseline TAG value for all the animals. Only one researcher was required to perform this study, and the use of physical restraint was not necessary due to the use of inhalatory isofluorane anaesthesia, resulting in lesser stressful experience for the animal and the researcher himself (Vachon et al., 2001). Inhalatory isofluorane anaesthesia was chosen due to its fast action and safety, which allows anaesthetic induction, oral cannulate and blood sampling in only five minutes per animal. In addition, it produces lower stress effect compared to other anaesthetic agents (Fitzner Toft et al., 2006; Altholtz et al, 2006). Indeed, it is known that stress effect strongly affects metabolic parameters such as cholesterol, glucose and TAGs (Ortolani et al., 2011). In addition, isofluorane had the interesting blocking action on the parasympathetic autonomic nervous reflex that leads to stop lingual lipase production, which is the major pre-duodenal lipase that contributes to triglyceride hydrolysis in rats (Loscar et al., 2004). This excluded the involvement of other lipases other than intestinal lipases. As far as we know there are not other methods that combine isofluorane anaesthesia, tail vein blood collection and TAG postprandial plasma level measurements.

Olive oil was included in treatment diets because triolein, its principal component, is a specific substrate for pancreatic lipase which is the only esterase class able to hydrolyze long-chain acylglycerols at the oil/water interface (Lowe et al., 1999). Moreover is considered safe since is broadly employed in animal and human nutrition. Lecithin and cholic acid were chosen as surfactant agents for their capacity to make homogenous emulsions and for being relatively safe. The results obtained in the present study shown that the use of lecithin alone resulted in elevated TAG plasma levels (two times higher then the other diet treatments at 120 minutes peak) than when combined with cholic acid. It could be argued that lecithin probably produces a better emulsion as well as smaller lipid particles which allow a better access of the lipase at the interface, which was likely to be inhibited by cholic acid. This is consistent with some *in vitro* studies reported that certain concentration of biliary salts could act as a barrier which prevent lipase to attach on lipid micelles (Borgström, 1977; Rouard 1978; Patton et al., 1981; Maldonado et al., 2011). Researches are in progress to investigate this hypothesis. Orlistat affected lipid digestion decreasing the TAG systemic uptake. Orlistat is the best known anti obesity drug (Suwailem et al., 2006) whose activity is based on an irreversible enzyme active site binding that leads to complete lipolysis inactivation in duodenum (Guerciolini et al., 1997). In the present study, inhibition occurred at 30 and 60 minutes, but at 120 minutes plasma TAG concentration dramatically increased, reaching even higher levels than the control at 180 minutes. These results were not consistent with previous reports that address a complete TAG plasmatic inhibition by Orlistat even at five hours after digestion (Han et al., 2005). We could speculate that influence of emulsion structure and stability might be responsible of such effect. Several studies support this by assessing how the emulsion type could itself inhibit lipase activity by decreasing space available on micelle where the enzyme could catalyze the TAG hydrolysis. This might be an important issue because it shows that emulsion composition could heavily impact the outcome of the lipid digestion.

CONCLUSION

On the light of these results we conclude that the method described in this study could be employed as short-term analysis to assess how different diets could affect TAG plasma levels on rats under anaesthesia. The current procedure is a fast and easy method that can be performed with minimal training. The entire time for manipulation/blood collection/administration does take no more than 5 minutes for each animal, which allows to test up to 5 animals at each time step.

REFERENCES

ALTHOLTZ, L. Y., FOWLER, K. A., BADURA, L. L., & KOVACS, M. S. (2006). Comparison of the stress response in rats to repeated isofluorane or CO_2:O_2 anaesthesia used for restraint during serial blood collection via the jugular vein. *Journal of the American Association for Laboratory Animal Science JAALAS, 45 (3), 17-22*.

BORGSTRÖM, B. 1977. The action of bile salts and other detergents on pancreatic lipase and the interaction with colipase. *Biochimica et Biophysica Acta*, *488* (3), 381-391.

CARRIÈRE, F., ROGALSKA, E., CUDREY, C., FERRATO, F., LAUGIER, R., & VERGER, R. 1997. *In vivo* and *in vitro* studies on the stereoselective hydrolysis of tri- and diglycerides by gastric and pancreatic lipases. *Bioorganic & Medicinal Chemistry*, *5* (2), 429-435.

DI MAIO, S. & CARRIER, R.L., 2010. Gastrointestinal contents in fasted state and post-lipid ingestion: *In vivo* measurements and *in vitro* models for studying oral drug

delivery. *Journal of Controlled Release*, 151 (2), p. 110-122.

EDDOUKS, M., LEMHADRI, A., & MICHEL, J.-B. (2005). Hypolipidemic activity of aqueous extract of *Capparis spinosa* L. in normal and diabetic rats. *Journal of Ethnopharmacology*, *98* (3), 345-350.

ELATAT, F., A. ANEJA, S. McFARLANE, & J. SOWERS. 2003. Obesity and Hypertension. *Endocrinology & Metabolism Clinics of North America* 32 (4), 823-54.

FITZNER TOFT M., PETERSEN, M. H., DRAGSTED, N., & HANSEN, A.K. 2006. The impact of different blood sampling methods on laboratory rats under different types of anaesthesia. *Laboratory Animals*, 40 (3), 261-274.

GUERCI, B., VERGES, B., DURLACH, V., HADJADJ, S., & PAUL, J. L. 2000. Relationship between altered postprandial lipemia and insulin resistance in normolipidemic and normoglucose tolerant obese patients. *International journal of obesity and related metabolic disorders journal of the International Association for the Study of Obesity*, *24* (4), 468-478.

GUERCIOLINI, R. (1997). Mode of action of Orlistat. *International journal of obesity and related metabolic disorders journal of the International Association for the Study of Obesity*, *23 (3)*, S12-S23.

HAN, L.-K., ZHENG, Y.-N., YOSHIKAWA, M., OKUDA, H., & KIMURA, Y. (2005). Anti-obesity effects of chikusetsusaponins isolated from *Panax japonicus* rhizomes. *BMC Complementary And Alternative Medicine*, *5*, 9.

KLEINSORGEN, R., & LIST, P. H. (1981). Emulsions with lecithin. *Pharmazie In Unserer Zeit*, *10* (1), 8-17.

LOSCAR, M., & CONZEN, P. 2004. Volatile anesthetics. *Der Anaesthesist*, *53* (2), 183-198.

LOWE, M.E. (1999). Assays for pancreatic triglyceride lipase and colipase. *Methods In Molecular Biology Clifton Nj*, *109*, 59-70.

MALDONADO-VALDERRAMA, J., WILDE, P., MACIERZANKA, A. & MACKIE, A. 2011. The role of bile salts in digestion. *Advances in Colloid and Interface Science*, *165* (1), 36-46.

ORTOLANI, D., OYAMA, L.M., FERRARI, E.M., MELO, L.L., & SPADARI-BRATFISCH, R. C. (2011). Effects of comfort food on food intake, anxiety-like behavior and the stress response in rats. *Physiology & Behavior*, *103*(5), 487-492.

PATTON, J.S., & CAREY, M.C. 1981. Inhibition of human pancreatic lipase-colipase activity by mixed bile salt-phospholipid micelles. *American Journal of Physiology*, *241*(4), 328-336.

REIS, P.M., RAAB, T.W., CHUAT, J.Y., LESER, M.E., MILLER, R., WATZKE, H.J., & HOLMBERG, K. (2008). Influence of surfactants on lipase fat digestion in a model gastro-intestinal system. *Food Biophysics*, *3* (4), 370-381.

RIZEK R., FRIEND B. & PAGE L. 1974. Fat in today's food supply. Level of use and sources, *J. Am. Oil Chem. Soc*. 51, 244–250.

ROUARD, M., SARI, H., NURIT, S., ENTRESSANGLES, B., & DESNUELLE, P. 1978. Inhibition of pancreatic lipase by mixed micelles of diethyl p-nitrophenyl phosphate and bile salts. *Biochimica et Biophysica Acta*, *530* (2), 227-235.

STAELS, B., & FONSECA, V.A. (2009). Bile acids and metabolic regulation, *American Diabetes Association, 32* (2), S237-S245.

SUWAILEM, A. K. 2006. Safety and mechanism of action of Orlistat (Tetrahydrolipstatin) as the first local antiobesity drug. *Journal of Applied Sciences Research*, *2* (4), 205-208.

UCHIYAMA, S., TANIGUCHI, Y., SAKA, A., YOSHIDA, A., & YAJIMA, H. 2011. Prevention of diet-induced obesity by dietary black tea polyphenols extract in vitro and in vivo. Nutrition Burbank Los Angeles County Calif, Elsevier *27*(3), 287-292.

VACHON, P., & MOREAU, J. P. 2001. Serum corticosterone and blood glucose in rats after two jugular vein blood sampling methods: comparison of the stress response. Contemporary topics in laboratory animal science *American Association for Laboratory Animal Science*, 40 (5), 22-24.

WANER, T. AND NYSKA, A. (1994). The influence of fasting on blood glucose, triglycerides, cholesterol, and alkaline phosphatase in rats. *Veterinary Clinical Pathology*, 23: 78-80.