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Analytical Methods

Development and validation of a dissolution test for lutein tablets and evaluation of intestinal permeability

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ABSTRACT

Lutein is a carotenoid with antioxidant activity that is present in various dosage forms. The bioavailability of carotenoid from oral dosage formulations depends on their release, dissolution and its permeability through the gastrointestinal tract. Here, a dissolution test was developed for evaluating formulations and the bioavailability was assessed. The test utilized a USP-apparatus II with rotations of 50, 75 and 100 rpm in water with P80 at 1, 2 and 5% (w/v). A non-everted rat intestinal sac model was used in conjunction to assess the intestinal permeability. The most discriminative conditions were 100 rpm in water with 2% polysorbate 80, which showed profile differences between two formulations. The intestinal permeation studies showed a lag-time and apparent permeability coefficient that were characteristic of highly permeable drugs. We suggest that a dissolution test can be an essential quality control tool for formulations containing compounds as lutein, although not mandatory by the regulation agencies.

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1. Introduction

The United States Food and Drug Administration (FDA) considers dietary supplements to be orally administered products that contain a dietary ingredient to augment or compensate dietary intake. These ingredients can include vitamins, minerals, herbs or other plants as well as amino acids, enzymes, organ tissues and metabolites (DSHEA, 1994). In 1994, a law was passed in the USA, known as the *Dietary Supplement Health and Education Act* (*DSHEA*), that declassified dietary supplements as medicines and, as a consequence, no longer required FDA registration prior to marketing. The responsibility for ensuring the effectiveness and safety of these products was transferred to the manufacturers along with the guarantee that their marketed products possess the benefits for human health alleged in their claims. In Brazil, regulation of dietary supplements is more stringent than in the USA and requires registration prior to marketing. In 1999, the responsible Brazilian Federal agency, ANVISA (National Health Surveillance Agency of Brazil), approved a number of regulations allowing a product containing substances present in the diet to be registered as a separate category of novel foods that provide health-enhancement. As such, these products are not classified as and do not adhere to regulatory controls enacted for dietary supplements. Furthermore, no regulatory legislation with regard to these products have been implemented to specifically assess physicochemical characteristics and bioavailability of active compounds in marketed products.

An example of a substance marketed with claims of functional properties, and approved by ANVISA, are carotenoids. They are a class of hydrocarbons that can be further divided into carotenes and xanthophylls substances, which are responsible for red or orange coloration of foods such as tomatoes and carrots, respectively (Garcia et al., 2012; Krinsky & Johnson, 2005). Typical diets contain around forty types of carotenoids. Of these, around twenty can be detected in plasma and tissue, whereas only two, zeaxanthin and lutein, accumulate in the retina (Widomska & Subczynski, 2014).

Lutein is a yellow pigment present in the macula lutea of human eyes and is classified as a xanthophyll carotenoid (Azqueta & Collins, 2012; Kijlstra, Yuan, Kelly, & Berendschot, 2012). Xanthophyll biosynthesis occurs only in plants, algae, bacteria and certain fungi (Kijlstra et al., 2012). Therefore, the source of these substances in the bloodstream is from dietary intake of dark green leafy vegetables, fruits, animal products such as eggs, or dietary supplements containing xanthophylls (Gellenbeck, Venzon, Lala, & Chavan, 2012; Kijlstra et al., 2012). Comprised of 40 carbon

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atoms and numerous double bonds, the molecular structure of lutein confers hydrophobicity and susceptible to breakdown by light, heat and oxidation, which contribute to its instability (Ambrósio, Campos & Faro, 2006).

Several studies have shown an association between the consumption of lutein and zeaxanthin with a reduction in chronic diseases such as age-related macular degeneration (Ahmed, Lott, & Marcus, 2005; Huang et al., 2013), anti-inflammatory properties that provide retinal neuroprotection (Sasaki et al., 2009) and a reduction in oxidative stress by eliminating reactive oxygen species (Stahl et al., 1998). After ingestion, lutein becomes incorporated into mixed micelles composed of bile acids, free fatty acids, monoglycerides and phospholipids. Lutein action depends on passive absorption by enterocytes and transportation by plasma lipoproteins (Ambrósio, Campos & Faro, 2006). Retinal tissue demonstrates greater uptake of lutein, which has enhanced abundance compared to plasma (Ahmed et al., 2005; Kijlstra et al., 2012).

In the Brazilian market, twenty-five products are registered as food, but marketed based on lutein content and antioxidant properties that are touted to have a beneficial impact on health. These formulations mainly consist of either a lutein base or ester contained in a capsule or pharmaceutical tablet form. The amount of lutein claimed in these products ranges from 2 to 10 mg per dosage and the daily recommended dosage suggested is one pharmaceutical unit per day (Anvisa, 2016). However, the quality of an oral dosage depends on its ability to release the active substance into aqueous media to facilitate its availability for gastrointestinal absorption (Azarmi, Roa, & Lobenberg, 2007; Davydova, Stippler, Jin, & Giancaspro, 2010).

The dissolution test is an important physicochemical quality control test to assess drugs during development. It has the potential to evaluate the *in vivo* performance of solid oral dosage forms since it assesses the release of the active substance into the dissolution medium over time (Shah et al., 1995; Dressman, Amidon, Reppas, & Shah, 1998; Siewert, Dressman, Brown, & Shah, 2003; Azarmi et al., 2007; USP, 2011, chap. 1092). Lutein is a lipophilic substance with low solubility in water and, in general, these characteristics reflect in low dissolution rate and oral bioavailability (Mitri, Shegokar, Gohla, Anselmi, & Muller, 2011). For these reasons, it is necessary to guarantee the bioavailability of lutein tablets by evaluating its release from dosage forms and its intestinal permeability. The aim of this work was to develop a discriminatory dissolution test to assess the release of lutein from oral tablets and predict the biopharmaceutical classification system to which lutein belongs. The classification of lutein in this study was based on its solubility and permeability during an *in vitro* assay using the non-everted rat intestinal sac model.

2. Methodology

2.1. Tablets of lutein

All tablets used in this study were purchased from drugstores in Brazil. The tablets obtained from different manufacturers of lutein were designated as B and D. Tablet B contained 2.0 mg of lutein and other constituents including: dibasic calcium phosphate dihydrate, calcium carbonate, magnesium oxide, ascorbic acid, niacin, beta-carotene, ferrous fumarate, zinc oxide, manganese sulfate monohydrate, calcium pantothenate, biotin, vitamin A acetate, vitamin E, vitamin D, anhydrous copper sulfate, vitamin K, pyridoxine hydrochloride, thiamine mononitrate, riboflavin, sodium selenate, folic acid, cyanocobalamin, chromium chloridehexahydrate, sodium molybdate, lactose, potassium iodide, microcrystalline cellulose, sodium croscaramellose, insoluble polyvinylpyrrolidone, hydroxypropyl methylcellulose, titanium dioxide, triacetin, polysorbate 80, indigotine, sunset yellow, red 40 and silicon dioxide. Tablet D contained 5.0 mg of lutein and the other constituents are: lactose, vitamin C, vitamin E beta-carotene, zeaxanthin, zinc, polyethylene glycol, riboflavin, copper, selenium, microcrystalline cellulose, polyvinylpyrrolidone, hydroxypropylmethylcellulose, hydroxypropylcellulose, stearic acid, ethyl cellulose, titanium dioxide, brilliant blue and silicon dioxide. The products were stored at room temperature and protected from light. All analyses were performed before the expiration dates of the tablets. Two batches from each manufacturer were selected.

2.2. Chemical products

The lutein chemical standard (Achemo, Hong Kong, China) was 90.03% pure. All solvents used in this study were of chromatographic grade and purchased from Tedia (Rio de Janeiro, RJ, Brazil). Additional reagents used were sodium lauryl sulfate (SLS), polysorbate (P80) and isopropyl alcohol, all of which were analytical grade and purchased from Vetec (Rio de Janeiro, RJ, Brazil). For all filtration procedures, 10 μm polyethylene filters were used and obtained from Water used in this study was purified using the Milli-Q water purification system from Millipore (Bedford, Massachusetts, USA).

2.3. Quantification by HPLC-DAD

The chromatographic method used was modified from Pintea, Bele, Andrei, and Socaciu (2003) and performed using a Merck-Hitachi Elite LaChrom liquid chromatograph (Darmstadt, Hesse, Germany) coupled to a diode-array detector (DAD L-2130), quaternary pump (L-2455), column oven (L-2350) and an autosampler (L-2200) through the control of EzChrom software. A Sunfire column (C_{18}; 4.6 × 250 mm; 5 μm particle size) from Waters (Milford, Massachusetts, USA) was coupled to a guard column from Kromasil (Bohus, Kungälv, Sweden) specific to the stationary phase. The mobile phase consisted of distilled water (A), acetonitrile (B) and ethyl acetate (C) and eluted as follows; 0–9 min: A (9–5%), B (81–45%) C (10–50%); from 9.1 to 15 min: A (5–1%), B (45–9%) C (50–90%); from 15.1 to 18 min: A (1–9%), B (9–81%), C (90–10%). The lutein standard solutions and samples were prepared in ethyl acetate and diluted in the mobile phase. All solutions were filtered through a 0.45 μm PVDF filters (Millex Millipore, São Paulo, SP, Brazil) before injection into the HPLC system. This quantification method was used to analyze the stability, solubility, dissolution and permeation of lutein in the isolated non-everted rat intestinal model.

2.4. Stability

Two saturated lutein solutions containing (1% P80 (w/v) or 1% SLS (w/v) in water were prepared, filtered through a 0.45 μm PVDF membrane and quantified by HPLC-DAD. The concentrations obtained were 0.5 μg/mL with 1% P80 (w/v) and 1.8 μg/mL with 1% SLS (w/v). Multiple 20 mL-aliquots from each solution were placed in tubes, closed, covered with aluminum foil and placed at 37 °C in a water bath. A 2 mL sample was collected from each tube after 24 h. The samples were filtered using 0.45 μm PVDF filters and quantified by HPLC-DAD.

2.5. Solubility

The solubility of lutein was determined in water, simulated gastric fluid (SGF; pH 1.2) and simulated enteric fluid (SEF; pH 6.8), according to USP 34 (USP, 2011, chap. 1092), in the absence of surfactant, and with the addition of P80 at 1, 2 and 5% (w/v).

A saturated solution was obtained by weighing approximately 15 mg of lutein and transferring to a 250 mL beaker containing 125 mL of pre-warmed medium at 37 °C. This solution was slowly stirred at 37 °C and a 5 mL aliquot was collected after 24 h. The test was performed in duplicate for each dissolution media tested. The samples were filtered through a 0.45 μm PVDF filters and quantified using HPLC-DAD.

2.6. Dissolution

The dissolution tests were performed using the Hanson Research SR6 (Chatsworth, CA, USA) dissolutor equipped with the USP dissolution apparatus II (paddle) and the auto Hanson Research collector. At least 6 tablets were used in each condition. A volume of 500 mL of dissolution medium (water) was maintained at 37 °C to test a variety of P80 and SLS concentrations with different rotations. From each condition, a 9 mL was collected at 0, 5, 15, 30, 45, 60, 90, 120, 150 and 180 min without replacing the volume removed. All aliquots were filtered using a porous polyethylene filter (10 μm; Hanson Research, Chatsworth, CA, USA) in the tubes of the automatic collector and subsequently by PVDF filters (0.45 μm). The samples were quantified using HPLC-DAD with standard curves of lutein and the straight-line equation to determine the amount of lutein dissolved from the tablets. The percentage of lutein dissolved during each collection time was calculated in relation to the amount labeled (considered 100%).

2.6.1. Dissolution kinetics

The results obtained from the dissolution profile (2% P80 (w/v) in water at 37 °C and 100 rpm) were used to evaluate the dissolution kinetics of lutein from the tablets. The straight-line equation and linear regression were used to determine the percentage of dissolved lutein as a function of time. The following kinetics models were used: zero order (time *x* amount of dissolved lutein), first-order (time *x* $\log_{10}$ of amount of dissolved lutein), Higuchi (square root of time *x* amount of dissolved lutein) and Korsmeyer-Peppas ($\log_{10}$ of time *x* $\log_{10}$ of amount of dissolved lutein). The mathematical model that best expressed the dissolution profile of lutein in the selected products was selected after linear regression and coefficient of determination analysis (R^2) (Costa & Lobo, 2001). Depending on coefficient of determination, suitable mathematical models to describe the dissolution profiles were determined.

2.6.2. Validation of the dissolution test

Dissolution tests, quantified using HPLC-DAD, were validated by evaluating a number of parameters including selectivity, linearity, accuracy and precision (ICH, 1996; Brazil, 2003, USP, 2011, chap. 1092). The validated dissolution method was the test with 2% of P80 (w/v) in water using the USP apparatus II and rotational speed of 100 rpm.

The selectivity of the method was determined using a placebo (containing all the constituents without lutein) for comparing to tablet D, lutein standard and the dissolution medium. Overlapping scans were obtained using the DAD detector at the beginning, middle and end of the detected signal of lutein in the chromatograms. Comparing the three-dimensional chromatograms of the placebo, dissolution medium and lutein standard allowed assessment of selectivity

The calibration curves were generated by serial dilution of the lutein stock solution. The standard was prepared in ethyl acetate followed by dilution into the mobile phase comprised of a mixture of ethyl acetate, acetonitrile and water (50:45:5) ranging between 0.5 and 20 μg/mL, which corresponds to a range of 5–200% of the working concentration (10 μg/mL). Linearity was determined by evaluating the equation of the line and the linear correlation coefficient after a linear regression of three calibration curves using five different concentrations and performed on different days.

The accuracy of the method was evaluated by contaminating the placebo tablet with a known amount of lutein standard. The standard solution was prepared using 95% ethanol (v/v) at a concentration of 200 μg/mL and added to the dissolutor vessels containing dissolved placebo in water containing 2% P80 (w/v) at 37 °C and rotated at 100 rpm. The percentage recovered was evaluated by adding different concentrations (20, 100 and 120%) of standard solution compared to working concentration (10 μg/mL), with three replicates each. After 1 h, 9 mL of each solution was collected and filtered through a 0.45 μm PVDF filters and quantified using HPLC-DAD. Accuracy was determined as the ratio between the average experimentally obtained concentration using the HPLC-DAD method and the corresponding theoretical concentration. The precision of the method was assessed by calculating the relative standard deviation (RSD) in triplicate and at the three different concentration levels (20, 100 and 120%). The precision of the method was verified for intra-day repeatability and intermediate precision measured on different days.

2.6.3. Statistical analysis of dissolution tests

The dissolution profiles of the tablets were evaluated by statistical analysis of the difference factor (f_1), the similarity factor (f_2) (Brazil, 2010; FDA, 1997; Shah, Tsong, Sathe, & Liu, 1998), One-way ANOVA with Tukey's post-test and Student's *t*-test. The statistical analyses were performed with GraphPad Prism (San Diego, CA, USA) version 5.0 for Windows.

2.7. Permeation of lutein in non-everted rat intestinal sac model

The method used was based upon approaches reported by Ruan and colleagues (Ruan et al., 2006). First, the donor solution was prepared by dispersing 150 mg of the lutein standard into 1.5 L of SEF containing 10% isopropyl alcohol (v/v) and 2% P80 (w/v). The donor solution was homogenized for 20 min at 37 °C. Subsequently, this solution was filtered using filter paper, followed by membrane filtration through 0.45 μm PVDF filters. The receptor solution consisted of phosphate buffer (pH 7.4) containing 10% (v/v) isopropyl alcohol and 2% (w/v) P80. Jejunum segments were collected from Wistar rats weighing 250–300 g after euthanasia with a 1:1 mixture of xylazine and ketamine hydrochloride (720 μL/kg). An abdominal incision was made to remove the jejunum, which was subsequently divided into 10 cm segments. Four jejunum segments were used in total. The cylindrical area (jejunum segment) had a radius (r) of 0.25 cm and height (h) of 10 cm. Cannulaes were inserted at the end of each jejunum segment and a peristaltic pump was connected to one end of each segment. The peristaltic pump pumped the donor solution at a flow rate of 1.2 mL/min. The other end remained free to allow exit of solution pumped into the jejunum segments. Each portion was placed in the perfusion chambers containing 10 mL of receptor solution and maintained at 37 °C by circulating water flow. Next, 1 ml aliquots were removed at the 0, 30, 60, 120, 180 and 240 min time points, and the volume was replaced across the receiving end. The collected samples were filtered through 0.45 μm PVDF filters and quantified using HPLC-DAD. At the end of each test, a methylene blue solution was passed through intestinal segments to verify that they maintained integrity. The results obtained from the permeation tests were plotted as mass average permeated (μg) *versus* time (min) and used to calculate the lag time (min). The apparent permeability coefficient (P_{app}) of lutein was calculated according to the following equation (Ruan et al., 2006):

$$P_{app} = (dQ/dt) \cdot (1/A \cdot C_0) \quad (1)$$

where, dQ/dt = (μg/s); A = area of the cylinder surface (cm^2); C_0 - = concentration in the donor solution (μg/mL).

All experimental procedures were approved by the Human and Animal Ethical Committee of the Center for Health Sciences/ Federal University of Rio de Janeiro (Registration Number: FARMACIA06).

3. Results and discussion

Lutein is a substance that presents an extreme thermal and oxidative lability (Ambrósio, Campos & Faro, 2006). These characteristics underlie the importance to evaluate its stability in dissolution mediums at the chosen temperature, 37 °C, during the development of tests designed to evaluate the process of dissolution. In addition, the use of surfactants is also essential since the mediums used are generally aqueous and lutein is hydrophobic. Surfactants alone can further accelerate the natural instability of hydrophobic substances. Therefore, the stability of lutein was assessed in water with the surfactants SLS and P80, two widely used in dissolution test mediums (Gowthamarajan & Singh, 2010; USP, 2011, chap. 1092). After 24 h at 37 °C, lutein was more unstable in the presence of SLS surfactant than in P80. The amount of lutein remaining was 60.75 ± 6.75% in SLS compared to 87.92 ± 8.34% with P80 (data not showed). A possible explanation for this observation is that SLS is an anionic surfactant while P80 is neutral in charge (Gowthamarajan & Singh, 2010). The charge carried by SLS could facilitate oxidation of lutein molecules and thus accelerate its degradation.

Further testing of lutein solubility was conducted in different mediums containing SGF or SEF with P80 to determine an optimal dissolution medium (Table 1). The differences in lutein solubility between the mediums in the absence of surfactant were insignificant and independent of the pH, as expected from previous findings in the literature (Ahmed et al., 2005; Mitri et al., 2011). Therefore, the use of surfactant was necessary to improve solubility of lutein. Another criteria for a proper dissolution medium is the sink condition, which is a 3-fold increase in the volume of medium required to obtain a saturated drug solution (Phillips, Pygall, Cooper, & Mann, 2012). However, if the test requires large amounts of surfactants or volumes of dissolution medium to solubilize a poorly soluble drug, the sink condition may not be feasible to maintain biorelevancy of the medium. In these cases, it is common to use a dissolution medium under non-sink conditions in order to guarantee better discriminative profiles (Tang, Khan, & Muhammad, 2001). Considering that tablet D contains 5 mg of lutein, its concentration in 500 mL of medium would be 10 μg/mL. Therefore, the aqueous SEF medium containing 2 or 5% P80 (w/v) and 1, 2 or 5% P80 (w/v) could be used in the dissolution test since the saturation concentration of lutein is higher than its concentration in the dissolution medium. Lastly, water was considered the most ideal medium since higher lutein solubility was achieved using the low and intermediate P80 surfactant concentrations.

Following the determination of the dissolution medium volume, conditions were evaluated for the solubilization of lutein. Using a single rotational speed of 75 rpm in the presence of the dissolution apparatus II (paddle), dissolution media containing 1 and 2% P80 or SLS (w/v) surfactants were tested. As shown in Fig. 1a, complete dissolution (>85%) of lutein was achieved in 1 and 2% SLS (w/v) in less than 30 min, For P80, complete dissolution required 90 min and a concentration of 2%. At the lower concentration of 1%, P80 did not completely dissolve lutein from tablets D. While the results showed a higher capacity of solubilization by SLS compared to P80 (Fonseca, Labastie, Sousa, & Volpato, 2009; Singla, Gupta, Kohli, & Singla, 2009; Simon, Borges, Cabral, & Sousa, 2013), the previous observation of lutein instability in SLS containing medium at longer incubations compelled the use of P80 as the surfactant for the lutein dissolution tests.

Considering that rotation speed can be a decisive factor that influences the dissolution process and directly reflects the dissolution activity over time, the common rotation speeds of 50, 75 and 100 rpm for the USP dissolution apparatus II were evaluated (USP, 2011, chap. 1092). After the initial testing at 75 rpm, the lower rotation speed 50 rpm was applied for aqueous media containing 1 and 2% P80 (w/v). Fig. 1b shows that dissolution was low and incomplete at either P80 concentration with the total percentage of lutein dissolved over a 180-min period measured at less than 20% of the level available in the tablets. Applying the higher rotation speed of 100 rpm proved more beneficial. Fig. 1c shows that complete lutein dissolution was obtained at 2 and 5% P80 (w/v) concentrations.

A dissolution test also requires that the RSD values of the first sampling time points does not exceed 20%, whereas for others, the sampling times were less than 10% (Brazil, 2010). The RSD values found in the first 5 and 10 min of testing with all rotations tested at each of the different concentrations of P80 were >20%, which defined a characteristic of this tablet formulation that can be considered as a quality deviation. In general, at increasing rotation speed, RSD values were observed to decrease, especially after 30 min of the test at all P80 concentrations used. However, only using aqueous media with 2 and 5% P80 (w/v) with a rotation at 100 rpm rotation provided RSD values that were lower than 10% after 30 min of dissolution test (Fig 1c).

The dissolution profiles obtained with 1 and 2% P80 (w/v) had an $f_1 < 15$ and an $f_2 > 50$, which were statistically similar at the three rotation speeds tested (Table 2). However, when 1% P80 (w/v) was used, it was found the dissolution profiles were incomplete at all the rotation speeds studied (Fig 1). This condition therefore, was not suitable for the dissolution test, potentially due the low solubility of lutein in this medium (11.02 μg/mL, Table 1). Since the lowest possible amount of surfactant is recommended in the dissolution test to ensure the discriminatory power of the medium (Gowthamarajan & Singh, 2010) and no differences were observed in dissolution profiles using 2 or 5% P80 (w/v) with the rotation speed of 100 rpm (Table 2), 2% P80 (w/v) was considered the ideal concentration for the dissolution tests. The dissolution parameters found in this study, suitable for the lutein dissolution test of tablet D were 2% P80 (w/v) in an aqueous medium at a temperature of 37 °C with a rotational speed of 100 rpm when using a USP dissolution apparatus II fitted with a paddle.

The dissolution test conditions developed were next applied to compare the dissolution profile of tablet B to tablet D and to assess the discriminatory capacity of the method. Fig. 2 shows the dissolution profiles obtained from tablets B and D. The release of lutein was observed to release more slowly from tablet B than tablet D with distinct profiles for each that reflected the values of $f_1 = 44.54$, $f_2 = 28.60$ ($p < 0.05$ by Student's *t*-test). After 90 min, a dissolution plateau was observed for tablet D, but not for tablet B. The slower lutein dissolution seen for tablet B could not be attributed to a saturation of the medium, since the measured saturating lutein concentration in water containing 2% P80 (w/v) was 20.40 μg/mL. The volume of medium used (500 mL) could

Table 1
Solubility at 37 °C of lutein in water, SGF and SEF without surfactants or in presence of varying concentrations of P80. Values are expressed in μg/mL.

Dissolution medium	Surfactant% (w/v)			
	WS	P80 1%	P80 2%	P80 5%
Water	0.00 ± 0.00	11.02 ± 0.30	20.40 ± 0.39	47.08 ± 2.36
SGF	0.10 ± 0.03	7.52 ± 0.21	9.02 ± 1.31	12.38 ± 0.99
SEF	0.11 ± 0.15	8.28 ± 0.49	18.17 ± 0.07	57.62 ± 0.52

Medium ± RSD; WS: without surfactant; P80: polysorbate 80; SGF: simulated gastric fluid; SEF: simulated enteric fluid.

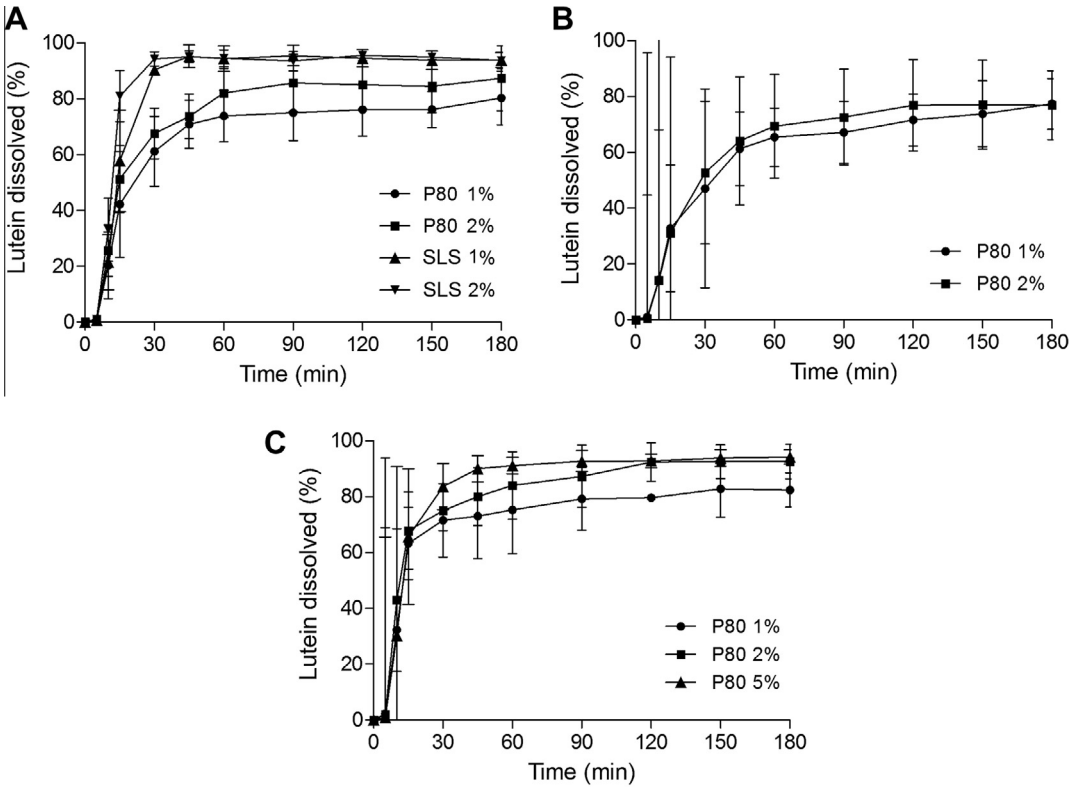

Fig. 1. Dissolution profiles of lutein obtained from tablet D in aqueous media with 1% and 2% P80 (w/v) or 1% and 2% SLS (w/v) at 37 °C under a rotational speed of 75 rpm (A), 50 rpm (B) and 100 rpm (C), using the dissolution apparatus USP II. Each point represents the mean result (n = 12) of lutein percentage dissolved at each time point.

Table 2
Statistical analysis of the dissolution profiles obtained with the tablet D in aqueous media with P80 1% and 2% (w/v) at 50 rpm and 75 rpm and P80 1, 2 and 5% (w/v) at 100 rpm in apparatus II from USP.

Medium (stirring speed)	f_1 (%)	f_2 (%)	Significance* P < 0.05
P80 1% (50 rpm) × 1% (75 rpm)	18.13	46.88	No
P80 1% (50 rpm) × 1% (100 rpm)	33.45	29.51	*
P80 1% (50 rpm) × 2% (50 rpm)	**9.13**	**66.38**	No
P80 1% (50 rpm) × 2% (75 rpm)	27.53	36.20	*
P80 1% (50 rpm) × 2% (100 rpm)	38.37	26.23	*
P80 1% (75 rpm) × 1% (100 rpm)	18.72	38.80	No
P80 1% (75 rpm) × 2% (50 rpm)	20.66	49.40	No
P80 1% (75 rpm) × 2% (75 rpm)	**11.49**	**53.91**	*
P80 1% (75 rpm) × 2% (100 rpm)	24.72	35.39	*
P80 1% (100 rpm) × 2% (50 rpm)	48.44	29.17	No
P80 1% (100 rpm) × 2% (75 rpm)	**13.14**	**49.53**	No
P80 1% (100 rpm) × 2% (100 rpm)	**10.04**	**51.49**	*
P80 2% (50 rpm) × 2% (75 rpm)	26.64	37.09	*
P80 2% (50 rpm) × 2% (100 rpm)	37.61	26.46	*
P80 2% (100 rpm) × 2% (75 rpm)	17.59	46.11	*
P80 5% (100 rpm) × 1% (50 rpm)	64.76	24.53	*
P80 5% (100 rpm) × 1% (75 rpm)	34.90	33.40	*
P80 5% (100 rpm) × 1% (100 rpm)	12.86	48.60	*
P80 5% (100 rpm) × 2% (50 rpm)	62.76	25.02	*
P80 5% (100 rpm) × 2% (75 rpm)	19.40	42.75	*
P80 5% (100 rpm) × 2% (100 rpm)	**8.17**	**57.85**	No

P80: polysorbate 80. f_1 = difference factor (0–15%); f_2 = similarity factor (50–100%); *One-way ANOVA - Tukey's test ($\alpha = 0.05$). No means P > 0.05 and * means P < 0.05. Bold: similar conditions with $f_1 < 15$ and $f_2 > 50$.

promote solubilization of more than five times the amount of lutein present in the dissolution medium with tablet B (4 μg/mL), considering the sink conditions.

We conclude that the slow release of lutein from formulation B is an intrinsic characteristic of this product. Formulations B and D are quite distinct in relation to excipient composition along with

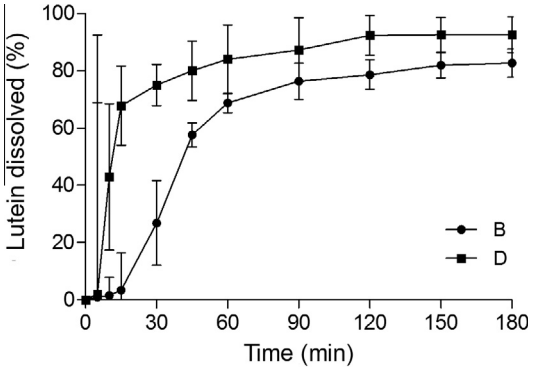

Fig. 2. Dissolution profiles of lutein obtained from tablet B (n = 12) and D (n = 12) in aqueous medium with 2% P80 (w/v) at 37 °C with a rotational speed of 100 rpm in the dissolution apparatus USP II. Each point represents the mean result of lutein percentage dissolved during at a given time. Statistical analysis: $f_1 = 44.54$, $f_2 = 28.60$ and $p < 0.05$ by Student's *t*-test.

the additional vitamins and minerals present in the tablets. Furthermore, tablet B has 2.0 mg of lutein, whereas D has 5.0 mg. It was desirable that the lutein dissolution test could discriminate between tablets B and D through differences in the lutein dissolution profiles. the results show that the dissolution method developed was discriminative and capable of detecting differences in release profiles of lutein from the two different tablet formulations. The dissolution profile of Tablet B followed first order kinetics ($R^2 = 0.99$; Appendix A), which are characteristic of immediate release pharmaceutical dosage forms that release of drug in proportion to the undissolved portion (Costa & Lobo, 2001). Tablet D followed the Higuchi kinetic model ($R^2 = 0.99$; Appendix A), which describes the release of drugs that are dependent on the square

Table 3
Accuracy and precision values of the test of dissolution of lutein using the method of quantification by HPLC-DAD.

Concentration (%)	Day	Recovery (%)	Average intraday recovery (%)	Average interday recovery (%)	Intraday (RSD%)	Interday (RSD%)
20	1	100.75	99.54	99.07	1.07	0.97
		98.87				
		99.01				
	2	98.09	98.60		0.58	
		98.59				
		99.12				
100	1	97.90	98.38	98.38	0.42	1.04
		98.78				
		98.47				
	2	96.34	98.38		1.66	
		99.02				
		99.79				
120	1	99.63	98.69	98.56	0.81	0.70
		98.53				
		97.92				
	2	97.77	98.36		0.49	
		98.69				
		98.63				

root of time due to diffusion in solid and semisolid matrices. Dosage forms following this model are characteristic of modified release systems (Costa & Lobo, 2001).

Dissolution tests must be validated to ensure that results obtained are reliable and reproducible. Therefore, our method was validated by evaluating the parameters of selectivity, linearity, precision and accuracy, as recommended by the USP and ICH (ICH, 1996; USP, 2011, chap. 1092). The selectivity of the method was observed by a comparative analysis of three-dimensional chromatograms obtained from the placebo, dissolution medium and tablet D dissolved in dissolution medium. No chromatographic signals corresponding to lutein were detected in the placebo and dissolution medium analysis. In the chromatogram of tablet D lutein, beta-carotene signals were detected and no interference was found (data not shown). The linearity of the method was tested in the range of 0.5–20 μg/mL. An average of the three standard curves using five concentrations was determined over different days and generated a coefficient of determination of 0.9999 with an angular coefficient of 967.657.3 ± 56.019.1 and an intercept of −56.713.3 ± 8.191. A wide concentration range was tested (corresponding to a range between 5 and 200% of working concentration) to ensure that small amounts of dissolved lutein were within the linear range of the quantification method. Furthermore, the method was proven to be both precise and accurate, since RSD intra and inter-day variability was less than 2% and the average recovery values ranged between 98 and 100% (Table 3).

In order for a drug to exhibit systemic action, it needs to dissolve properly in the gastrointestinal tract followed by permeation through the intestine wall. Various studies model permeation through uptake by Caco-2 cells and have reported lutein absorption values of 6% (Gleize et al., 2013), 7% (During, Hussain, Morel, & Harrison, 2002) and 10% (O'Sullivan, Ryan, & O'Brien, 2007). While Caco-2 cells are a common, assessable model, a more comprehensive system for studying intestine permeability is the use of rodent intestinal sections. To date, this model has not been used for evaluating lutein permeability. A previous study using rat jejunum segments evaluated the P_{app} of natural products and compared them to P_{app} of drugs, thereby estimating the fraction absorbed *in vivo* (Ruan et al., 2006).

In the present study, lutein permeation tests were performed using the non-everted rat intestinal sac model. From the results shown in Fig. 3, the P_{app} was calculated to be 1.13×10^{-5} cm/s by evaluating the average concentration of the permeated lutein *versus* time. The P_{app} found for lutein in this test is comparable to the coefficient found for both theophylline and caffeine (Ruan et al., 2006), drugs that are highly absorbed *in vivo*. Therefore, it is assumed that lutein was highly absorbed in this model.

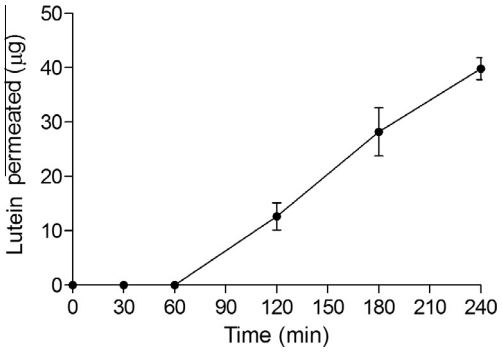

Fig. 3. Intestinal permeation of lutein in isolated rat intestine showing the mean ± SEM (μg/mL) *versus* time (minutes) permeated concentration. N = 4 segments of jejunum.

Considering that lutein is highly lipophilic with a log P of 7.8 (Shanmugam et al., 2011), the results shown here are consistent with its properties, including a high level of permeability in isolated rat intestine. Highly hydrophobic substances permeate the intestinal membrane by passive diffusion. However, data in the literature indicates that intestinal absorption of lutein is variable since it is poorly soluble, and subsequently dissolution is highly dependent on the matrix in which lutein is formulated (Shanmugam et al., 2011). Gastrointestinal transit velocity, the amount of time the drug and absorption site are in contact, is another important factor to consider and can impact drug bioavailability. The permeation test results here indicated that lutein had a lag time of 60 min, which was the time required for lutein to begin being absorbed by rat jejunum segment. Thus, the speed of intestinal transit also impacts the bioavailability of lutein. The results of low solubility of lutein in aqueous media, together with high permeability shown by its P_{app} value suggests that lutein belongs to class II of the biopharmaceutical classification system. Drugs of this class have similar properties and their *in vitro* dissolution is the rate limiting step in absorption since the absorption rate is greater than the dissolution rate (Amidon, Lennernas, Shah, & Crison, 1995). In addition, the dissolution profiles obtained were found to depend on the type of formulation, once again indicating the importance of discriminatory dissolution methods.

4. Conclusion

Based on the results of stability and solubility of lutein, a dissolution test was developed for lutein tablets. Optimal conditions were determined to include an aqueous medium containing 2% P80 (w/v) with rotational speed of 100 rpm using the USP dissolution apparatus II. The method was found to be discriminative and could detect differences in lutein release profiles from two different tablet formulations procured from different manufacturers. The dissolution test was validated for specificity, linearity, accuracy and precision suggesting it would be appropriate for intra-day and inter-day analysis. In permeation tests using rat intestine, the P_{app} value obtained for lutein was characteristic of highly permeable substances. The results demonstrating that lutein has low solubility in aqueous media, coupled with its high permeability suggests that lutein is a class II drug according to the biopharmaceutical classification system. The difference in lutein dissolution profiles obtained for commercial tablets B and D indicates that the formulation has a major effect on the release of carotenoid from dosage forms. Overall, the results validate the effectiveness of the described dissolution test in discerning formulation characteristics that effect an essential step in lutein release that could be implemented as a method of quality control for lutein tablets performance to assess the potential bioavailability of lutein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.04.081.

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