Enhanced Oral Bioavailability of Astaxanthin with Droplet Size Reduction

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Astaxanthin is a natural xanthophyll abundantly found in *Haematococcus pluvaris* with strong antioxidant property and it is found to be useful in many biological functions. Recent scientific findings indicate that astaxanthin shows low oral bioavailability due to its lipophilicity. However, the influence of emulsion droplet size on astaxanthin bioavailability is yet to be studied. This study was performed to investigate the effects of emulsion droplet size reduction on the bioavailability of astaxanthin emulsion through oral administration. *In vivo* study was carried out to evaluate the rate and extent of absorption of macro and nanosized emulsion an oil solution formulation. The nanosized emulsion shows increased C_{max} and $AUC_{0-\infty}$ and decreased T_{max} values with a reduction of the emulsion globule size. The $AUC_{0-\infty}$ of nanosized compared to macrosized emulsion and oil solution was increased by 110% – 140% and 140% – 200% respectively.

Keywords: astaxanthin, bioavailability, droplet size reduction

Introduction

Astaxanthin is a naturally occurring carotenoid that belongs to the xanthophyll group with excellent antioxidant properties. Astaxanthin is *de novo* synthesized by bacterium belonging to genus *Paracoccus*, yeast (*Xanthophyllomyces dendrorhous*), green algae (*Haematococcus pluvaris* and *Chlamydomonas nivalis*) and Adonis plants (Goswami *et al.*, 2010). Astaxanthin is also found in crustaceans, salmon and trout (Kurashige *et al.*, 1990; Clark *et al.*, 2000). They accumulate astaxanthin through the food chain and metabolic conversion. Recent scientific findings indicate that astaxanthin showed quenching activity for singlet oxygen and inhibition of lipid peroxidation compared to β -carotene and vitamin E by 40 and 1000 times, respectively (Kurashige *et al.*, 1990; Shimidzu 1996). It has been found to provide many essential biological functions, such as protection against lipid-membrane peroxidation of essential polyunsaturated fatty acids and proteins, DNA damage and ultra violet light effects. It also plays an important role in immunological defence and prevention of age-related macular degeneration (AMD) (Martin *et al.*, 2003).

Animals lack the ability to synthesize the carotenoids

de novo, and can be supplied with carotenoids, that are biosynthesized by microorganisms or by algae and plants, through their dietary intake (Olaizola and Huntly, 2003). Commercially astaxanthin is produced from both synthetic and natural sources. The primary and abundant natural source of astaxanthin for commercial uses is microalgae *Haematococcus pluvaris*. It synthesizes astaxanthin from the carotenoids, lycopene or phytoene when exposed to extreme environmental conditions and ultraviolet light (Lorenz and Cysewski, 2000).

Astaxanthin like other carotenoids is very lipophilic compound which recorded low oral bioavailability. It varies from less than 10% for raw uncooked vegetables to more than 50% in oily solutions or in synthetic commercial formulations (Olson, 1994). As a fat soluble material, astaxanthin follows the same intestinal absorption path as dietary fat. Astaxanthin absorption involves several steps from the breakdown of the food matrix to the release of astaxanthin into the lumen of the gastrointestinal tract (GIT) through their incorporation into lymphatic lipoproteins (Furr and Clark, 1997). This includes mechanical and chemical disruption of the food matrix, dispersion in lipid emulsion particles, solubilization into mixed bile salt micelles, movement across the unstirred water layer adjacent to the microvilli, uptake by the enterocyte, and incorporation into lymphatic lipoproteins

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(Furr and Clark, 1997). Any disruption at any point along this chain contributes to low absorption of astaxanthin.

Thus far, very limited information is available concerning the improvement of astaxanthin oral bioavailability. Most of the studies were focused on improvements of the carotenoid such as β -carotene. These include studies on oil-in-water emulsions (Amar *et al.*, 2003), nanodispersions (Tan and Nakajima, 2005) and oil-in-water nanoemulsions (Yuan *et al.*, 2008). These studies show an impressive result in improving β -carotene bioavailability. The only research done so far on the improvement of astaxanthin bioavailability was performed by Odeberg *et al.* (2003), who proved that the bioavailability of astaxanthin could be increased by incorporating the drug into an emulsion form. This positive result was attributed to the presence of surfactants in the emulsion, which increases the surface area for the release of astaxanthin from the vehicle. Emulsion itself also mimics the formation of bile salt micelles (which are formed during lipolysis and facilitate absorption of lipophilic compounds) and thereby increases the solubility of astaxanthin (Odeberg *et al.*, 2003).

By formulating astaxanthin in the emulsion form, which is considered as lipid-based formulation, there is a high chance for the drug to gain access to the systemic circulation via lymphatic route which can avoid hepatic first-pass metabolism. As a continuous improvement from the studies done by Odeberg *et al.* (2003), it has become an interest to us to investigate the influence of emulsion droplet size on the bioavailability of astaxanthin.

Materials and Methods

Materials Astareal extract 10FC grade (an oil extract containing 10% w/w of standardized astaxanthin derived from *Haematococcus pluvialis*) was purchased from Fuji Chemical Industry (Nakaniikawa, Toyama, Japan) Industry (Nakaniikawa, Toyama, Japan), lecithin (L- α -phosphatidylcholine, Type IV-S $\geq$ 30% TLC (thin layer chromatography) - P3644) was purchased from Sigma (St Louis, Missouri, USA) while Tween 80 (polyoxyethylene (20) sorbitan monooleate) was purchased from Zulat Pharmacy (Selangor, Malaysia). Pure palm olein was purchased from FFM Berhad (Selangor, Malaysia). The water used was obtained from Elga Pure Lab (Marlow Buck, UK) water system. All other chemicals used were of analytical grade unless otherwise stated.

Methods

1) Preparation of astaxanthin oil formulation Astaxanthin oil (which contained 2% w/w of standardized astaxanthin) was prepared by diluting astaxanthin extract 10FC grade in pure palm olein. The mixture was then mixed ho-

mogeneously for 5 min at 50 rpm. This formulation was used as the base of the emulsion and reference formulation of this study.

2) Preparation of astaxanthin emulsion formulation

Astaxanthin emulsion was formulated as per method developed by Meor Mohd Affandi *et al.* (2011). Astaxanthin oil (16%) was mixed with purified water (80% w/w) containing Tween 80 and lecithin as the emulsifier (4% w/w). To produce macro sized emulsion, the premix was homogenised by High Speed Homogeniser (HSH) (Silverson Homogeniser, UK) at 9000 rpm for 5 min to give oil droplet average size of 8.9 μ m. Nanosized emulsion was produced by further homogenization of macrosized emulsion with High Pressure Homogeniser (HPH) (model APV 1000, APV Systems, Albertslund, Denmark) at 800 bar for 5 cycles. Astaxanthin oil preparation was used as the reference formulation of this study. Table 1 simplified the ratio of component and treatment used in each of the formulations.

3) Bioavailability study protocol All experiments were performed according to Universiti Teknologi MARA (UiTM) guidelines of experimental animal care and approved by UiTM Animal Research and Ethics Committee. Male Sprague-Dawley rats weighing approximately 200 g were used. The rats were maintained at controlled temperature and relative humidity on a 12-hour light-dark cycle. They were fasted for 12 h before formulation administration and for 3 h after dosing. This study was a cross-over study and the rats were divided into 3 groups of 3 rats each, to which all the 3 different formulations were given orally. The washout period of one week between each treatment was implemented (Table 2). Water was freely available throughout the duration of the experiments.

Blood sampling Blood samples were taken from the tail at 0, 1, 2, 3, 4, 6, 8, 10, 12, 24, 48 and 72 h. All samples were collected in 5 mL heparin tubes and were centrifuged at 14000 rpm for 20 min. Plasma separated was transferred into the eppendorf microcentrifuge tube and stored at -20°C until further analysis.

Analytical method Analysis of astaxanthin was performed using direct injection method with simple deproteinizing process developed by Meor Mohd Affandi *et al.* (2012). The detector was operated at a wavelength of 474 nm. A Phenomenex HPLC column from Phenomenex, USA fitted with synergi 4 μ m Hydro-RP 80A (150 $\times$ 4.6mm) and a refillable guard column (Phenomenex, USA) was used for the chromatographic separation. The mobile phase comprised methanol:water:ethyl acetate (82:8:10 (v/v)). Analysis was run at a flow rate of 1.5 mL/min and the samples quantified using area under the curve (AUC) by isocratic mode.

Plasma sample and control plasma were thawed at room

Table 1. Astaxanthin formulations : Ratio of component and treatment used in each of the different formulations.

Formulation	Astaxanthin Extract	Palm Olein	Water	Surfactant (Tween 80 + Lecithin)	High Speed Homogeniser (HSH)	High Pressure Homogeniser (HPH)	Droplet size (μm)
Reference (Astaxanthin Oil)	2%	98%	—	—	—	—	—
A (macrosized emulsion)	2%	14%	80%	4%	9000 rpm 5 min	—	8.9 ± 0.014
B (nanosized emulsion)	2%	14%	80%	4%	9000 rpm 5 min	800 bar 5 cycles	0.128 ± 0.015

Table 2. Three-way cross over design schedule with 3 rats in each group.

Group (3 rats / group)	Week		
	1st	2nd	3rd
1	A	B	C
2	B	C	A
3	C	A	B

A : Oil formulation B : Macrosized emulsion
C : Nanosized emulsion

temperature under minimal exposure to light. The plasma was then treated using the following procedure: 100 μL plasma was measured into an eppendorf microcentrifuge and deproteinised using 200 μL of mixture of ethanol and tetrahydrofuran (1:9). The mixture was vortexed for 2 min using a vortex mixer and centrifuged at 14000 rpm for 20 min (Eppendorf, USA). After centrifugation, a 50 μL of the clear supernatant was then injected into the HPLC system. The astaxanthin was eluted using an isocratic gradient with methanol:water:ethyl acetate (82:8:10) as a mobile phase. Astaxanthin was detected at 474 nm and was eluted at 4.1 min.

Pharmacokinetics calculations The following kinetic parameters were estimated for astaxanthin: C_{max} (maximum observed plasma concentration), T_{max} (the time to reach maximum concentration), $\text{AUC}_{0-\infty}$ (area under the curve calculated to infinity which reflects the total amount of drug that reaches the systemic circulation) and $t_{1/2}$ (elimination half-life). C_{max} , T_{max} , and $t_{1/2}$ were read directly from the observed concentration versus time data. A linear-log trapezoidal method was used when calculating $\text{AUC}_{0-\infty}$.

Statistical analysis In this experiment, assumption was made that C_{max} , $t_{1/2}$ and $\text{AUC}_{0-\infty}$ for astaxanthin, estimated to be normally distributed when log transformed. In order to achieve the primary objective of this study which is to find formulations which were superior, a comparison of means was made between groups using one-way ANOVA followed by Student's t-test, with 95 % confidence interval. Statisti-

cal analyses were carried out using the SPSS version 17.0. The whole data collection on in drug formulation experiment were done at least in triplicate.

Results and Discussion

In order to investigate the effects of different lipid based formulations and droplet size emulsion formulations on the bioavailability of astaxanthin after oral administration, the macro and nanosized emulsion formulations were given orally to the rats and astaxanthin in oil was used as reference. The plasma profiles of astaxanthin in adult Sprague Dawley rats following oral administration of macrosized emulsion, nanosize emulsion and oil solution of astaxanthin were compared.

It is clearly presented in Figure 1 that astaxanthin administered in macrosized and nanosized emulsion represents significantly greater improvement of drug absorption than astaxanthin in oil. This result agrees with the findings of Odeberg *et al.* (2003) who reported that the absorption of astaxanthin can be enhanced when surfactants with the right combination of lipophilic and hydrophilic are added in the formulation. As also presented in Figure 1, astaxanthin administered in nanosize shows the highest astaxanthin plasma concentration followed by the macrosized emulsion and oil solution.

Fig. 1. Rat plasma concentration versus time profile of nanosized emulsion, macrosized emulsion and oil solution of astaxanthin after oral administration of 80 mg/kg astaxanthin formulation (n = 9).

Effects of Emulsion droplet size on astaxanthin bioavailability Different types of lipid based formulations play an important role in enhancing the absorption of astaxanthin (Odeberg *et al.*, 2003). Both emulsion formulations, namely nanosize and macrosized show a significant increase in astaxanthin absorption compared to oil solution. Thus far, studies on astaxanthin have only focused on the effects of lipid based formulations on astaxanthin absorption (Odeberg *et al.*, 2003). Therefore, it has become an interest to study the effects of emulsion droplet size on astaxanthin bioavailability.

Effects of emulsion droplet size in $C_{\max}$, $AUC_{0-\infty}$ and $T_{\max}$ As presented in Figure 1, astaxanthin administered in nanosize shows the highest astaxanthin plasma concentration followed by the macrosized emulsion and oil solution. As shown further in Table 3, decreasing the emulsion droplet size resulted in significant increase in $C_{\max}$. Nanosized astaxanthin emulsion mean $C_{\max}$ was 698.7 ± 38.7 ng/mL, compared to that of macrosized astaxanthin emulsion which was 465.1 ± 43.0 ng/mL and oil solution which was 313.3 ± 12.9 ng/mL. Statistically, the difference in mean $C_{\max}$ values, were significant ($P < 0.05$) when the nanosize emulsions were compared to the macrosized astaxanthin emulsions and astaxanthin oil solution. The 95% confidence interval of the logarithmic transformed $C_{\max}$ value of nanosized astaxanthin emulsion over those of macrosized emulsion and oil solution was found to lie between 1.4 to 1.5 and 2.1 to 2.4, respectively.

It was also observed that the decreasing of emulsion droplet size resulted in a significant increase in $AUC_{0-\infty}$.

The mean value of $AUC_{0-\infty}$ nanosized astaxanthin emulsion was 20625 ± 5072 h.ng/mL compared to those of macrosized astaxanthin emulsion which was 15859 ± 1682 h.ng/mL and oil solution which was 11484 ± 2412 h.ng/mL. Statistically, the difference in $AUC_{0-\infty}$ of nanosized astaxanthin emulsion was significant ($P < 0.05$) when compared to $AUC_{0-\infty}$ of macrosized astaxanthin emulsion and astaxanthin oil solution. The 95% confidence interval of the logarithmic transformed $AUC_{0-\infty}$ value of nanosized astaxanthin emulsion over those of macrosized emulsion and oil solution was found to lie between 1.0 to 1.4 and 1.4 to 2.0, respectively.

The significant increase of $C_{\max}$ and $AUC_{0-\infty}$ by the decreasing of emulsion droplet size clearly shows that the absorption of astaxanthin can be enhanced when the emulsion droplet size is reduced. Smaller oil droplet size will increase the surface area of the drug and improve its solubility behaviour as well as its permeation. Moreover, smaller oil droplet size will not delay gastric emptying time and this will result in faster drug absorption. These statements are in agreement with the $T_{\max}$ values which are reduced with the decrease of emulsion droplet size. Nanosized emulsion achieved the shortest $T_{\max}$ which was 2.8 ± 0.73 h followed by macrosized emulsion (4.3 ± 0.50 h) and oil solution (5.5 ± 0.66 h). Statistically, the difference in $T_{\max}$ of nanosized astaxanthin emulsion, is significant ($P < 0.05$) when compared to $T_{\max}$ value of macrosized astaxanthin emulsion and oil solution.

Effects of emulsion droplet size on K_e and $t_{1/2}$ Table 4 indicates that the K_e and $t_{1/2}$ of three different sizes of emulsion oil droplet. The drug elimination for all formulations follow the first order kinetics where drug decreases exponen-

Table 3. Individual numerical value of $C_{\max}$, $T_{\max}$ and $AUC_{0-\infty}$ of three types of formulation tested on nine.

Subject	Oil Solution			Macrosized emulsion			Nanosized emulsion		
	$C_{\max}$ (ng/mL)	$T_{\max}$ (h)	$AUC_{0-\infty}$ (h.ng.mL)	$C_{\max}$ (ng/mL)	$T_{\max}$ (h)	$AUC_{0-\infty}$ (h.ng.mL)	$C_{\max}$ (ng/mL)	$T_{\max}$ (h)	$AUC_{0-\infty}$ (h.ng.mL)
1	325.08	5.0	11302.83	499.60	4.0	17719.50	697.08	3.0	20730.78
2	330.16	4.0	13563.82	429.55	4.0	16587.34	653.33	2.0	25505.29
3	315.76	5.0	6804.70	429.55	4.0	12349.32	640.84	2.0	24732.22
4	311.61	5.0	11037.66	403.74	4.0	14214.22	732.93	3.0	13251.19
5	309.40	6.0	14204.42	437.39	5.0	15137.53	682.69	4.0	15671.24
6	294.92	6.0	10702.20	497.28	4.0	16201.53	746.32	3.0	16383.32
7	330.01	6.0	10910.69	459.73	5.0	16977.00	694.47	3.0	26937.56
8	301.36	6.0	14656.05	531.82	4.0	16587.53	751.51	2.0	24931.84
9	301.91	6.0	10175.88	497.22	5.0	16961.73	689.22	3.0	17482.05
Mean	313.36	5.4	11484.25	465.10	4.3	15859.52	698.71	2.8	20625.05
SD	12.924	0.667	2412.654	43.008	0.5	1682.619	38.733	0.726	5072.939
CV%	4.1	12.4	21.0	9.2	11.6	10.6	5.5	25.9	24.60

CV: Coefficient of variation, SD: Standard Deviation

Table 4. Individual numerical value of $t_{1/2}$ and K_e of three types of formulation tested on nine.

Subject	Oil Solution		Macro-sized emulsion		Nanosized emulsion	
	K_e (h^{-1})	$t_{1/2}$ (h)	K_e (h^{-1})	$t_{1/2}$ (h)	K_e (h^{-1})	$t_{1/2}$ (h)
1	0.0268	25.9	0.023	30.7	0.022	31.4
2	0.0257	27.0	0.035	20.1	0.022	31.3
3	0.0569	12.2	0.029	23.8	0.024	28.5
4	0.0255	27.2	0.022	31.9	0.021	33.1
5	0.0226	30.7	0.019	37.0	0.031	22.7
6	0.0207	33.5	0.026	26.6	0.033	20.9
7	0.0328	21.2	0.019	35.9	0.015	46.5
8	0.0208	33.4	0.023	30.7	0.021	33.2
9	0.0210	33.0	0.024	29.1	0.029	23.6
Mean	0.0281	27.1	0.024	29.5	0.024	30.1
SD	0.01147	6.95876	0.00499	5.43425	0.00576	7.723903
CV%	40.8	25.7	20.5	18.4	23.7	25.67

CV: Coefficient of variation, SD: Standard Deviation

tially with time and $t_{1/2}$ was recorded at 30.1 ± 7.7 h, 29.5 ± 5.4 h and 27.1 ± 6.9 h for nanosized emulsion, macro-sized emulsion and oil solution, respectively. These results did not deviate much from the previous published studies regarding the pharmacokinetics of astaxanthin, which reported the half-lives at 21 ± 11 h (Osterlie *et al.*, 2000) and 15.9 ± 5.3 h (Odeberg *et al.*, 2003). The results also show that the size of emulsion globule does not influence K_e and $t_{1/2}$ value.

Conclusion

This study confirmed that the bioavailability of astaxanthin could be enhanced in rats with the reduction of oil droplet size. The nanosized emulsion shows a significant increase in C_{max} and $AUC_{0-\infty}$ value followed by macro-sized emulsion and oil solution. Conversely the T_{max} value was reduced with the reduction of emulsion globule size. The kinetics of astaxanthin is described as a one-compartment model with a half-life of 29.7 ± 7.4 h, 28.9 ± 5.4 h and 27.5 ± 6.6 h for nano-sized and macro-sized emulsion and oil solution, respectively. In this present study, we focused on the bioavailability improvement of astaxanthin by using rats as a subject. Since the usage and application of astaxanthin for human consumption has emerged in the past few years in the nutraceutical field, it is becoming interesting to investigate the performance of the astaxanthin nanoemulsion formulated in this study on human subjects.

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