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Comments from the Editors and Reviewers:

Reviewer #1: The manuscript describes an HPLC method for β-carotene quantification. Moreover, the authors developed a new type of formulation based on in situ forming gels loaded with solid drug dispersions. I believe that the results presented here are novel and are appropriate for publication in this journal. The manuscript is well written and the results are presented and discussed appropriately. Simple analytical methods with lower LoD/LoQ are always interesting and therefore I recommend this paper for publication just after some minor revision. Please see specific comments:

1. I think that using "Solvent A" and "Solvent B" can be confusing. I suggest the authors to use Methanol and SGF (Simulated Gastric Fluid).

2. The authors present the results suggesting that the obtained values are in line with ICH guidelines. These results are great but could you please put them into context. Could you please compare them briefly with previously developed methods for beta-carotene quantification? Just a couple of lines in the discussion will be enough.

3. I think that the mathematical modelling section for the release data should be expanded slightly. Korsmeyer-Peppas model will provide an exponent value that will define the release mechanism. After figuring that out you can apply different models for each type of release mechanism (ie. Higuchi for diffusion). Please have a look here for guidance: https://doi.org/10.1002/jps.23774

Reviewer #2: This piece of work is describing the development and validation of a new HPLC method to quantify β-carotene as per the guidelines from ICH. Moreover, Enggi et al. developed a novel approach combining solid

dispersion and floating gel in situ to improve not only the solubility, but also to sustain the release of βcarotene. Overall, the experiments were well designed, and the results were well discussed. Moreover, this manuscript presents an interesting work within the field of pharmaceutical analysis. Therefore, this is a good and valuable piece of work for the potential readers of this journal, which should be accepted in its present form.

Reviewer #3: The paper described an intersting approach to improve the release and absorption of beta-carotene and a new validated HPLC-UV method for its detection and quantification.
However, the manuscript needs major revision before the publication.
- A deep English revision is needed
- The title is too long and not clear
- too many figures and Tables. Select only the most important in the text and add the others to supporting material-
- beta carotene is not a drug, but a pro-vitamin. So, avoid the use of "drug" because is very confusing.

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HPLC-UV method validation for quantification of β-carotene in the development of sustained release supplement formulation containing solid dispersion-floating gel in situ.

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HPLC-UV method validation for quantification of β-carotene in biorelevant media: Application to drug content analysis and *in vitro* release behavior studies in a sustained release supplement formulation containing solid dispersion-floating gel *in situ*.

Cindy Kristina Enggi, Fitrah Mahardika, Delly Mayari Devara, Mesakh Diki Saputra, Nurfadilla Wafiah, Andi Dian Permana*

Faculty of Pharmacy, Hasanuddin University, Makassar, 90245, Indonesia

***Corresponding author:**

Andi Dian Permana

Faculty of Pharmacy, Hasanuddin University, Indonesia

Email: andi.dian.permana@farmasi.unhas.ac.id

ABSTRACT

Despite the health benefits of β-carotene, its activity has been hampered by poor aqueous solubility and low oral bioavailability. Therefore, it is crucial to develop a new approach to overcome these problems. In this study, we developed a dry powder supplement comprising a combination approach of solid dispersion and floating gel *in situ* of β-carotene to enhance the solubility and achieve sustained release behavior. Here, we validated an HPLC method to quantify β-carotene as per the guidelines from ICH. The analytical method was validated in methanol and Fasted-State Simulated Gastric Fluid (FaSSGF) to determine β-carotene in recovery and *in vitro* release studies, respectively. A simple HPLC method using Xselect CSH™ C18 column (Waters, 3.0 x 150 mm) with the particle size of 3.5 µm was validated with 100% acetonitrile as mobile phase. The calibration curves were found to be linear with LLOQ values < 3 ng/mL. Importantly, the method was accurate and precise without carry over effect and successfully applied to determine β-carotene concentration in the drug content and *in vitro* drug release from floating gel *in situ* laden with solid dispersion formulations. The sensitivity of method obtained here offers a wide potential use in various applications in drug delivery systems.

Keywords: β-carotene, solid dispersion, floating gel *in situ*, HPLC-UV, validation

1. INTRODUCTION

β-carotene is one of the vital biological agents that is extensively contained in fruits, vegetables and many plants. This bioactive compound is lipophilic in nature and has fascinated significant consideration from researchers, particularly in pharmaceutical and food science due to its benefits for human health benefits [1,2]. Several investigations have proven that the nutritional consumption of β-carotene may decrease the risk of several diseases. This compound is able to protect human cells from the damaging effects of radiation of UV [3]. Acting as a strong antioxidant, β-carotene is able to remove excessive reactive oxygen species produced in the body. This action makes β-carotene show the ability to prevent degenerative illnesses including cardiovascular diseases, infectious diseases and numerous kinds of cancer. In its application, it can be utilized as an active pharmaceutical compound, in cosmetics and in foods [4].

Nevertheless, despite the benefits mentioned previously, the poor water solubility and poor oral bioavailability limit its application [5]. Additionally, this compound has been found to be unstable with oxygen, light and heat [4]. Accordingly, it is necessary to find an approach that can improve the water solubility and bioavailability of this compound.

Currently, different approaches have been tried to solve the solubility issues of lipophilic bioactive compounds [6,7]. Amongst all the approach, solid dispersion method is a favorable approach for the improvement of water solubility of lipophilic compounds [8]. To prepare this approach, several hydrophilic polymers, namely polyvinylpyrrolidone (PVP), hydroxypropylmethyl cellulose (HPMC), hydroxypropyl cellulose (HPC), and cyclodextrin derivates are commonly used as vehicles [9,10]. Furthermore, to control the release of β-carotene, the selection of final dosage form is crucial.

Gastroretentive drug delivery system (GRDDS) is a method that has been developed to overcome the bioavailability problem occurring from hydrophobic compounds. These approaches are intended to retain the active compounds in the gastrointestinal and sustain the release of the drug. This system would be beneficial to be used as a food supplement for daily used of β-carotene. One of GRDDSs is the floating raft system (GFRS), well-known as *in situ* floating gel, which would be well preserved in the stomach because of the ability of this system to float in the acid environment [11]. Several biopolymers such as sodium alginate, gum and many others have been utilized to formulate this approach due to several benefits, including low price, accessibility, biodegradation, low toxicity and sustainability. Sodium alginate is a biodegradable polymer that swells in normal pH aqueous environment and forms a gel in acid aqueous environment [12,13]. Therefore, this polymer could potentially be used as a pH

sensitive polymer in *in situ* floating gel formulation. Importantly, this system has been developed into dry form, which could be potentially further formulated into dry powder for food supplement reconstituted with water prior to its consumption.

In the formulation process, analytical method is one of the important parts. This part would allow the formulation scientists to detect and quantify the analytes of interest in the *in vitro* release study, especially for sustained release dosage form. With respect to β-carotene analysis, generally, the analytical method was carried out using high performance liquid chromatography (HPLC) with a reversed phase C-18 columns and detection at visible part of spectra [3,14]. Several studies have developed analytical methods to quantity β-carotene in different conditions. However, the limit of quantification values obtained were all above 50 ng/mL [3,14–16]. Considering that the oral dose of β-carotene is considerably low (around 14-45 mg daily) [17] in the high volume of body human fluid, accordingly, in controlled and sustained formulation development, which would release the compound in low concentration, it is extremely important to develop a new analytical method which allows us to detect β-carotene in very low concentration.

In the present work, we report a simple and sensitive HPLC method to quantify and analyze β-carotene. The analytical method was validated as per the guidelines from International Council for Harmonization (ICH). The validated method was successfully applied to determine β-carotene of palm oil extract. Furthermore, in this study, the β-carotene of palm oil extract was formulated into solid dispersion which was further incorporated into *in situ* floating gel system. Finally, the validated method was again applied to quantify the drug content and the drug released in the *in vitro* release study.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

β-carotene, HPLC grade acetonitrile, poly(vinylpyrrolidone) PVP (30 kDa) and polyethylene glycol (PEG) 6000 were obtained from Sigma-Aldrich Pte Ltd (Singapore). Xselect CSH™ C18 column with 3.0 x 150 mm and particle size of 3.5 µm was purchased from Waters (Dublin, Ireland). All other chemicals used in this study were of analytical grade.

2.2. Instrumentation and Chromatographic Conditions

Chromatographic analyses were performed in a HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan) coupled with photo diode array detector. The separation of analyte of interest was achieved using Xselect CSH™ C18 column (Waters, 3.0 x 150 mm) particle size of 3.5

μm at 35 °C. Acetonitrile 100% was used as mobile phase which was degassed for 15 minutes prior to use in bath sonicator. The analysis run time was 15 minutes and β-carotene were detected at 452 nm. The flow rate and the injection volume were 0.5 mL/min and 50 μL, respectively. Peaks of β-carotene in the extract, formulation and drug release medium were recognized by observing the retention time in each sample and were matched to the retention time of β-carotene authentic standards.

2.3. Preparation of stock solutions, calibration standards and quality control samples

The preparation of β-carotene stock solutions was carried out by dissolving 5 mg of standard β-carotene in 100 mL of methanol. The stock solution was further diluted in suitable solvents to achieve calibration standard solutions with the concentrations between 1 ng/mL and 1000 ng/mL. The standard solutions were prepared in two solvents, namely methanol (solvent A) and Fasted-State Simulated Gastric Fluid (FaSSGF) neutralized with NaOH 1 M to pH 7 (solvent B). Briefly, FaSSGF was made by solubilizing 1.999 g of NaCl in 1 L purified water, altered to pH 1.5 with HCl 1 M [18]. Additionally, three quality control solutions were prepared by diluting the stock solution solutions of β-carotene in solvent A and B to achieve low quality control (3 ng/mL), medium quality control (350 ng/mL) and high-quality control (750 ng/mL). All calibration standard and quality control solutions were made in three replications.

2.4. Method validation

The HPLC method developed previously was validated according to International Conference on Harmonization (ICH) guidelines [19,20]. Several parameters, namely selectivity, linearity, limit of detection (LOD), lower limits of quantification (LLOQ), carry-over, dilution integrity, precision and accuracy were all evaluated.

2.4.1. Selectivity

The selectivity test was carried out by analyzing the sandard solutions of β-carotene in solvent A and solvent B. The chromatogram of the standard solutions were compared to the blank solvent A and solvent B, as well as blank formulation of solid dispersion and floating gel *in situ* prepared in methanol.

2.4.2. Linearity, LOD and LLOQ

The linearity of the analytical method was assessed by constructing the calibration curve consisting the analytes concentration (x axis) and the peak area (y axis). The calibration was

considered to be linear if correlation coefficient (r^2) were above 0.998 [21]. Furthermore, the LOD and LLOQ values were calculated using the following Equations:

$$LOD = 3.3\sigma/S \quad \text{(Equation 1)}$$

$$LLOQ = 10\sigma/S \quad \text{(Equation 2)}$$

Where σ = the SD of the response of the data and S = the slope of the calibration curve.

2.4.3. Carry-over and dilution integrity

To investigate the presence of carry-over during the analysis, two blank samples were injected directly following the injecting of HQC solutions. The carry over was considered when the area of the blank samples were more than 20% of the area of the LLOQ solutions [22].

To evaluate the dilution integrity, β-carotene solutions were prepared at 2500 ng/mL in solvent A and solvent B. Furthermore, the solutions were diluted 5 and 10 times with appropriate solvents and the area of analytes were observed.

2.4.4. Accuracy and precision

Accuracy and precision were evaluated for the intra-day and the inter-day. The intra-day evaluation was carried out in the same day, while the inter-day evaluation was performed in three consecutive days. Following this evaluation, the relative standard deviation (RSD, %) and relative errors (RE, %) were calculated to represent the values of accuracy and precision, respectively. In these evaluations, samples at LLOQ, LQC, MQC and HQC were used.

2.5. Formulation of solid dispersion

Solid dispersions of β-carotene were prepared using a solvent evaporation method [8]. Briefly, 500 mg β-carotene, PEG (500 mg) and PVP (500 mg) were accurately weighed and dissolved in 25 mL chloroform. The solution was then evaporated at 50ºC to obtain solid dispersion powder. The process was carried out in the dark condition to protect the degradation of β-carotene. Subsequently, the solid dispersion was crushed in a mortar and pestle before being passed through a 45μm sieve. The obtained powder was packed in an airtight container, stored in a desiccator prior to further investigations [23].

2.6. Formulation of solid dispersion loaded dry floating gel in-situ

Dry floating gel in situ formulations were prepared by mixing solid dispersion (equal to 100 mg of β-carotene), sodium alginate (2 g), HPMC K100 (2 g), sodium bicarbonate (1.5 g), calcium carbonate (1.5 g) in the mortar. The mixture was stored in airtight containers before

evaluation. As control, floating gel *in situ* containing pure β-carotene and solid dispersion containing pure β-carotene were also prepared.

2.7. Application of the method

2.7.1. Determination of β-carotene recovery in solid dispersion and floating gel in situ formulations

The recoveries of β-carotene in solid dispersion and floating gel in situ formulations were determined by dissolving each formulation which was equal to 10 mg of β-carotene in 100 mL methanol in bath sonicator for 1 hour. All samples were centrifuged at 12,000 rpm for 15 minutes. The supernatant was collected and diluted appropriately prior to analysis using validated HPLC method.

2.7.2. Determination of β-carotene saturation solubility in solid dispersion formulation

Four different media were used to determine the saturation solubility of β-carotene following the formulation of solid dispersion, namely purified water, FaSSGF, phosphate buffer saline (PBS) solution pH 6.8, and PBS solution pH 7.4. In this study, an excessive amount of solid dispersion was added into each medium. The mixture was stirred at 100 rpm 37°C for 24 hours. Finally, the mixture was centrifuged at 12,000 rpm for 15 minutes. The supernatant was collected and diluted appropriately prior to analysis using validated HPLC method.

2.7.3. Determination of β-carotene in the in vitro drug release studies

The dry floating gel *in situ* formulation was reconstitute in water prior to the *in vitro* release study. The release profile of β-carotene from solid dispersion and floating gel *in situ* formulations were evaluated using USP Dissolution Apparatus II at 37°C at 50 rpm. An aliquot of each formulation which was equal to 15 mg β-carotene was placed in a dissolution vessel filled containing 900 ml of FaSSGF. Samples (5 mL) were taken at predetermined time (15 minutes, 30 minutes, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h and 24 h) from dissolution media and were replaced with 5 mL fresh media. The concentration of β-carotene was determined using validated HPLC method. The kinetics of dissolution profile were assessed using various mathematic models, as follows [18]:

$$Zero\ Order\ Kinetics: C_t = C_0 + k_0 t \quad (3)$$

$$First\ Order\ Kinetics: \ln C_t = \ln C_0 + k_1 t \quad (4)$$

$$Higuchi\ Model: C_t = k_H \sqrt{t} \quad (5)$$

$$Korsmeyer - Peppas\ Model: C_t = k_{KP}t^n \quad (6)$$

$$Hixson - Crowell\ Model: C_t^{1/3} = C_0^{1/3}k_{HC}t \quad (7)$$

Where C_t is the concentration of β-carotene released at time t, C_0 is the initial amount of β-carotene in FaSSGF (at t=0), k_0 is constant of zero-order kinetics, k_1 is constant of first-order kinetics, k_H is constant of Higuchi model, k_{KP} is constant of Korsmeyer-Peppas model, and k_{HC} is constant of Hixson-Crowell model. All values were calculated using DD-solver software [24]

2.8. Statistical Analysis

The mean, SD RSD and RE were calculated using Microsoft® Excel® 2016 (Microsoft Corporation, Redmond, USA. Furthermore, all data were displayed as mean ± SD. GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA) was utilized to statistically analyze all the data. *p* values < 0.05 were considered as significant differences.

3. RESULTS AND DISCUSSION

3.1. Selectivity of HPLC method

The primary aim of this study was to develop and validate analytical method of β-carotene in sustained release formulation. RP-HPLC was preferred for the analysis of the drugs. This method is an analytical method which principally separates chemical substances depending on their polarity. In addition, this method is also able to identify drugs rapidly and accurately and can be used in the purification of compounds.

Taking into considerations regarding the forthcoming utilizations of the methodologies developed, some other aspects that may affect separation were assessed in a selectivity evaluation, as illustrated in Figure 1. In this step, blank solvent A, blank solvent B, blank solid dispersion and blank floating gel *in situ* formulations were injected and analyzed using the similar method of β-carotene analysis. Specifically, two solvents were used in this study, namely solvent A (methanol) and solvent B (Fasted-State Simulated Gastric Fluid (FaSSGF) neutralized with NaOH 1 M to pH 7). Solvent A was used in the drug content analysis in solid dispersion and floating gel *in situ* formulations. Furthermore, solvent B was used to analyze β-carotene in the *in vitro* drug release studies. It was observed that the retention time of β-carotene was 9.74 minute. The results showed that no interfering peaks were found in the chromatograms of β-carotene with blank solvent A, blank solvent B, blank solid dispersion and

blank floating gel *in situ* formulations. This showed that the method presented selectivity, with an excellent resolution of the peaks and the analytes of interest were not influenced by the appearance of expected endogenous or impurities compounds in *in vitro* studies.

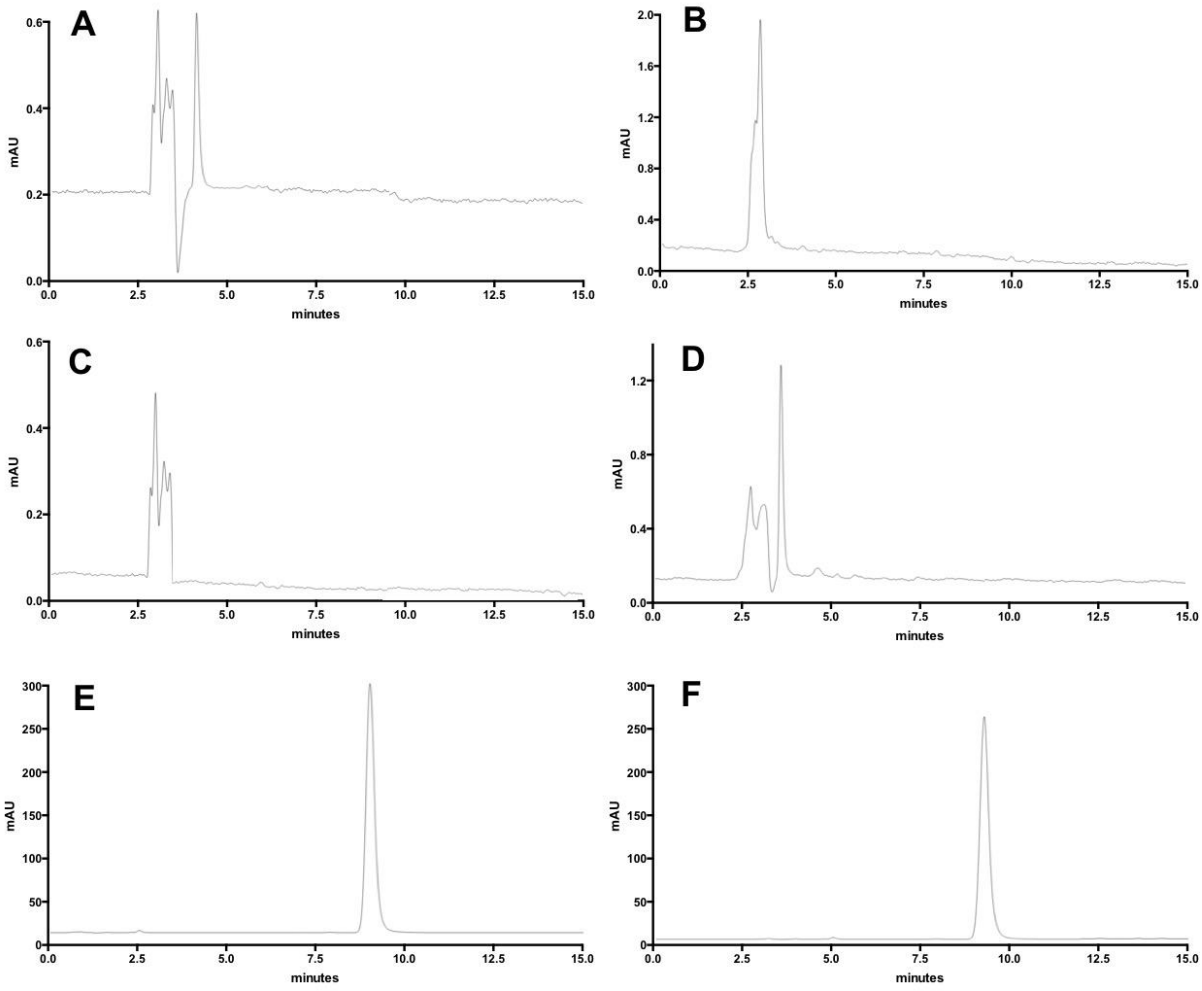

Figure 1. Representative HPLC-UV of chromatograms of blank solvent A (**A**), solvent B (**B**), blank solid dispersion (**C**), blank floating gel in situ (**D**), β-carotene in solvent A (**E**) and β-carotene in solvent B (**F**).

3.2. Linearity, LOD, and LLOQ

In this step, the calibration curves in solvent A (methanol) and solvent B (FaSSGF) neutralized with NaOH 1 M to pH 7 were constructed. It was obtained that r^2 values of the calibration curves in solvent A and solvent B were 0.999 and 0.997, respectively, Therefore, it could be considered that the curves were linear. Furthermore, the LOD values were 0.7 ng/mL for solvent A and 0.9 ng/mL for solvent B. The LLOQ values for solvent A and solvent B were 2.5 ng/mL and 2.7 ng/mL. Importantly, LLOQ values in our study were considerably lower

compared to those values reported in the previous study [3,14–16]. Accordingly, our method was found to be more sensitive to the current methods available.

3.4. Carry-over and dilution integrity

In the analytical process, especially when using HPLC, carry-over effect should be avoided in order to ensure that the quantification of high-concentration samples did not affect the response of other samples. In this investigation, after the injection of β-carotene in HQC concentrations, there was no peak observed in the blank solvents. Thus, the method developed did not show any carry-over effect.

Additionally, it is important to bear in mind that the concentration of β-carotene released from sustained release formulation in the dissolution media could not be forecasted. Therefore, the dilution integrity was critical to be performed. After the dilution of high concentration of β-carotene in both solvents with the dilution factors of 5 and 10, the percentages of β-carotene recovery were between 97.89 ± 8.04% and 99.18 ± 9.32 %. Furthermore, the precisions were found to be from 9.32% to 11.28%. Considering that the precision should be ±15%, the dilution integrity assessment of this study fulfilled the prerequisite from ICH. Consequently, these findings implied that the concentration of β-carotene which were above the calibration standard solutions concentration can be determined by appropriate dilution.

3.3. Accuracy and precision

Inter-day and intra-day of accuracy and accuracy of the developed HPLC method were assessed using β-carotene solutions with LLOQ and QC concentrations in solvent A and solvent B. Table 1 and Table 2 show %RSD and %RE presenting precision and accuracy of the method. It was found that inter-day and intra-day precision evaluation resulted in %RDS values which were in the range of 6.41% – 12.18% and 6.15% – 13.01%, respectively. Moreover, the accuracy evaluations exhibited %RE of -6.33 – 7.78 for inter-day evaluation and -7.20 – 9.20 for inter-day evaluation, respectively. Considerably, according the limits established by ICH (15%), the developed HPLC to determine β-carotene was found to be accurate and precise.

Table 1. Precision and accuracy data for the HPLC method for quantification of β-carotene in solvent A (means ±S.D., *n*=3).

Intra-day Precision and Accuracy				
Replication	**Concentration added (ng/mL)**	**Concentration found (ng/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.5	2.54 ± 0.21	8.27	1.60
	3	3.09 ± 0.29	9.39	3.00
	350	344.12 ±28.32	8.23	-1.68
	750	768.43 ± 81.98	10.67	2.46
2	2.5	2.38 ± 0.19	7.98	-4.80
	3	2.89 ± 0.26	9.00	-3.67
	350	363.82 ± 30.01	8.25	3.95
	750	773.19 ± 72.81	9.42	3.09
3	2.5	2.64 ± 0.22	8.33	5.60
	3	2.81 ± 0.18	6.41	-6.33
	350	338.82 ± 29.91	8.83	-3.19
	750	739.17 ± 88.21	11.93	-1.44
Inter-day Precision and Accuracy				
Day	**Concentration added (ng/mL)**	**Concentration found (ng/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.5	2.32 ± 0.18	7.76	-7.20
	3	2.87 ± 0.32	11.15	-4.33
	350	371.02 ± 30.17	8.13	6.01
	750	736.91 ± 47.39	6.43	-1.75
2	2.5	2.73 ± 0.27	9.89	9.20
	3	3.02 ± 0.35	11.59	0.67
	350	354.42 ± 29.93	8.44	1.26
	750	749.91 ± 48.31	6.44	-0.01
3	2.5	2.39 ± 0.17	7.11	-4.40
	3	3.21 ± 0.28	8.72	7.00
	350	329.03 ± 40.43	12.29	-5.99
	750	737.92 ± 73.18	9.92	-1.61

Table 2. Precision and accuracy data for the HPLC method for quantification of β-carotene in solvent B (means ±S.D., *n*=3).

Intra-day Precision and Accuracy				
Replication	**Concentration added (ng/mL)**	**Concentration found (ng/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.7	2.91 ± 0.23	7.90	7.78
	3	3.19 ± 0.32	10.03	6.33
	350	332.98 ± 39.93	11.99	-4.86
	750	765.18 ± 48.54	6.34	2.02
2	2.7	2.66 ± 0.18	6.77	-1.48
	3	3.13± 0.28	8.95	4.33
	350	355.03 ± 30.12	8.48	1.44
	750	766.01 ± 59.03	7.71	2.13
3	2.7	2.69 ± 0.21	7.81	-0.37
	3	3.02± 0.32	10.60	0.67
	350	329.87 ± 40.18	12.18	-5.75
	750	744.93 ± 55.47	7.45	-0.68
Inter-day Precision and Accuracy				
Day	**Concentration added (ng/mL)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.7	2.88 ± 0.24	8.33	6.67
	3	2.92 ± 0.38	13.01	-2.67
	350	382.39 ± 33.21	8.68	9.25
	750	765.18 ±65.03	8.50	2.02
2	2.7	2.91 ± 0.32	11.00	7.78
	3	3.09 ± 0.19	6.15	3.00
	350	342.91 ± 32.91	9.60	-2.03
	750	768.32 ± 60.03	7.81	2.44
3	2.7	2.81 ± 0.18	6.41	4.07
	3	3.22 ± 0.28	8.70	7.33
	350	348.93 ± 44.03	12.62	-0.31
	750	783.21 ± 65.43	8.35	4.43

As previously discussed, solid dispersion is a favorable approach the solubility issue of β-carotene. Compared to other approach, this method is a comparatively simple technique formed by the interaction between hydrophilic vehicles and hydrophobic compounds. Amongst numerous polymers used in solid dispersion preparation, PVP and PEG have been found to be the most utilized polymeric compounds. The application of these polymers in the solid

dispersion preparation has been widely discussed in numerous studies [25–29]. Several methods have been used to prepare solid dispersion from polymeric matrix, namely solvent evaporation, kneading, melting, lyophilization and spray-drying. Solvent evaporation was chosen due to its simplicity because it does not need a specific machine to prepare the solid dispersion.

PEG is a semi-crystalline polymer of ethylene oxide with specific characters, namely its ability to produce solid drug solutions, rapid solidification level, low melting point, non-hazardous, and reasonably low price [30]. Furthermore, PVP is an amorphous polymer possessing an excellent biocompatibility and high solubility in water. This polymer has been widely utilized in solid dispersion preparation due to the capability in stabilizing amorphized drug crystals [31]. In this study, β-carotene solid dispersion was prepared using the combination of PVP K-30 and PEG 6000.

Following the formulation of solid dispersion, the saturation solubilities of β-carotene were examined in on four media with various pH values. Specifically, the validated HPLC method developed in this study was used to assess the saturation solubility values. The results showed that our solid dispersion formulation was able to improve the saturation solubility of β-carotene in the extract and standard forms, when compared to pure extract and standard β-carotene. Table 3 shows the saturation solubility of β-carotene in various system evaluated in this study. In the solid dispersion system, the hydrophilic carrier plays role as a solubilizer agent. The presence of these carrier could enhance the wettability of hydrophobic compounds, leading to improved solubility in aqueous environment [32].

Table 3. Saturation solubility of β-carotene in solid dispersion formulation compared to pure β-carotene (means ±S.D., *n*=3).

Media	Saturation solubility values	
	Pure β-carotene	**Solid dispersion**
Purified water	3.29 ± 0.23 ng/mL	28.11 ± 2.52 µg/mL
FaSSGF	2.93 ± 0.22 ng/mL	23.87 ± 2.32 µg/mL
PBS pH 6.8	3.75 ± 0.19 ng/mL	29.98 ± 2.09 µg/mL
PBS pH 7.4	3.79 ± 0.34 ng/mL	34.32 ± 3.17 µg/mL

To sustain the release of β-carotene from solid dispersion formulation, it was further incorporated into floating gel *in situ* system. In this study, the formulation of gel was due to the crosslink reaction between alginate and calcium ion. *In situ* delivery system using ion crosslinking-based to physically retain the formulation in the upper part of the gastric provides

a distinctive approach in the formulation a controlled release dosage form in the liquid state. As a calcium source, calcium carbonate was used in the formulation. It is non-soluble compound which did not react with the alginate in the formulation. When reaching the acid environment, calcium carbonate released the calcium ion and reacted with alginate, forming ion crosslinking-based gel [33]. Furthermore, the addition of $NaHCO_3$ would help formulation to float on the surface of gastric solution. Finally, HPMC is a hydrophilic polymer used to control the release of β-carotene from final formulation product. Figure 2 exhibits the representative the floating gel *in situ* formulation in the FaSSGF media, showing that the formulation swelled in floated in the media.

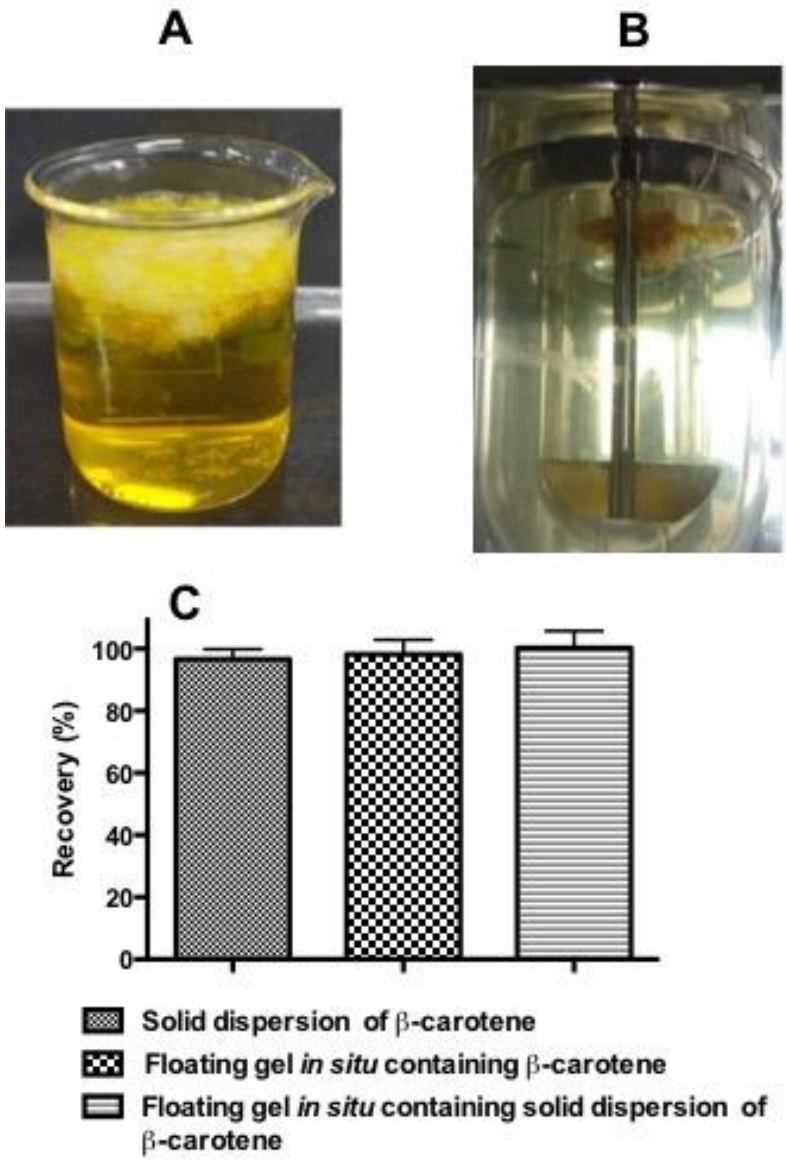

Figure 2. Representative images of floating ability of floating gel *in situ* formulation containing solid dispersion of β-carotene (A and B). The percentage of recovery of β-carotene in various formulations (n=3, mean ± SD).

The further application of the HPLC method validated in this study was to determine the β-carotene content in the solid dispersion containing β-carotene, floating gel *in situ* formulation containing β-carotene, as well as floating gel *in situ* formulation containing solid dispersion of β-carotene. Figure 2 presents the percentage of β-carotene contents in all formulations tested. The results showed that all recovery values were between 96.54% and 100.34%. This indicates that the excipients and method applied in the formulation process did not affect the β-carotene concentration in the formulations. Additionally, this also implied that the method applied produced the homogeneous formulation. ICH has recommended the acceptable recovery percentage was 95 – 105% [34], indicating that all formulations tested fulfilled the requirement.

Furthermore, the validated HPLC method was applied to determine the cumulative *in vitro* release of β-carotene from solid dispersion formulation, as compared to only β-carotene extract. Figure 3 exhibited the comparison of β-carotene release profile from solid dispersion and from its extract form. After 24 h, solid dispersion formulation released 74.65 ± 9.56% β-carotene. Instead, only 16.75 ± 2.45% of β-carotene released from pure β-carotene. This indicated that the solid dispersion formulation could improve the release percentage of β-carotene by three times higher than pure β-carotene. There was considerably higher of the release of β-carotene following solid dispersion formulation ($p < 0.003$) compared to pure β-carotene. As previously discussed, the enhanced release profile of β-carotene in the solid dispersion system was due to the presence of the hydrophilic carrier which improved the wettability, resulting in enhanced *in vitro* release profile [32]. It was also crucial to investigate the release mechanism of β-carotene from solid dispersion formulation. This was performed by fitting the release profile to different mathematic kinetic models. Following the calculation, the release profile of the formulation resulted in correlation coefficient of 0.59, 0.90, 0.49, 0.87 and 0.82 for Zero-order, First-order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell, respectively. With these values in mind, the most suitable model for β-carotene release from solid dispersion was First-order model, indicating that the release profile of β-carotene depended on the concentration of β-carotene in the polymeric matrixes [6,35].

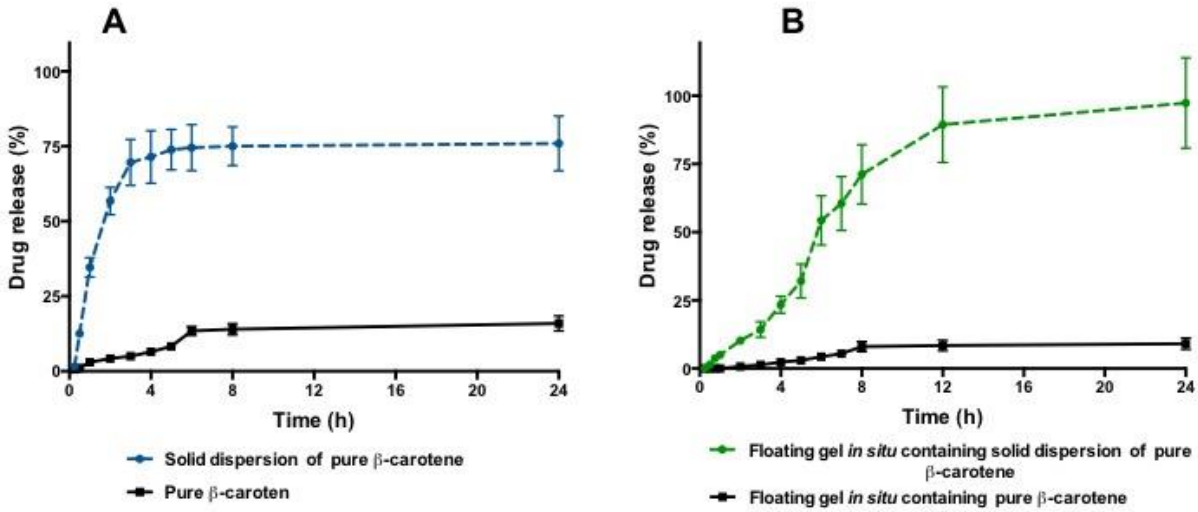

Figure 3. *In vitro* dissolution study of β-carotene from solid dispersion formulation compared to pure β-carotene (n = 3, mean ± SD) (A). *In vitro* release profile of β-carotene from floating gel *in situ* formulations (n = 3, mean ± SD) (B).

Finally, the *in vitro* release behavior of β-carotene following the incorporation of solid dispersion was evaluated using the validated HPLC method, as shown in Figure 3. According to the results obtained, the formulation of solid dispersion of β-carotene into alginate-based floating gel *in situ* formulation was able to sustain the release over 24 h, resulting in 98.54 ± 10.32% of the drug released percentage. On the other hand, the formulation of pure β-carotene t into floating gel *in situ* significantly decreased ($p < 0.05$) the release of β-carotene with only 9.24 ± 2.32% release after 24 h. Moreover, following being fitted to mathematic kinetic models, it was found that the correlation coefficient values were observed to be 0.85, 0.89, 0.43, 0.97 and 0.88 for Zero-order, First-order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell, respectively. The incorporation of solid dispersion into floating gel *in situ* formulation resulted in the release mechanism following Korsmeyer-Peppas model. This model has been generally found in a polymeric drug delivery system. As the main compound of this approach was polymeric material, the mechanism of β-carotene from this system was hypothetically based on swelling-relaxation of the polymer [36,37].

Based on the results found in this study, the combination of solid dispersion and floating gel *in situ* was able to improve both the solubility and sustain the release of β-carotene for 24 h. Essentially, the validated HPLC method developed in this study was successfully applied to quantity β-carotene in the drug loading and *in vitro* release study of our combination approach. However, in forthcoming steps, the release profile of β-carotene following this approach should

be carried out in a suitable *in vivo* animal model. To achieve this, the analytical method for *in vivo* studies must also be developed and validated.

4. CONCLUSION

The current investigation reports a sensitive an analytical method to quantiy β-carotene from a sustained release formulation. The analysis process was carried put using HPLC which was validated according to ICH guidelines. The analysis was performed using 100% acetonitrile in Xselect CSH™ C18 column with 3.0 x 150 mm internal diameter and particle size of 3.5 µm at 35 °C. Specifically, the HPLC method was validated in 2 different solvents, namely methanol (solvent A) and Fasted-State Simulated Gastric Fluid (FaSSGF) neutralized with NaOH 1 M to pH 7 (solvent B). Solvent A was used in the drug content analysis and solvent B was used in the drug release study. The method was found to be linear, selective, sensitive, accurate and precise. In this study, we also successfully developed an approach to combine solid dispersion and floating gel *in situ* to improve the solubility and sustain the release of β-carotene. Finally, the validated method was applied to quantify β-carotene in the drug content and *in vitro* drug release, showing that the formulation possessed uniform content and excellent recovery with sustained release behavior following 24 hours. Lastly, the development of *in vivo* analytical method should be carried out and applied in *in vivo* pharmacokinetic studies.

Declaration of Competing Interest

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTION

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript

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