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Optimized determination of lycopene in canine plasma using reversed-phase high-performance liquid chromatography☆

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Abstract

An isocratic high-performance liquid chromatographic method with detection at 472 nm was developed, optimized and validated for the determination of lycopene in canine plasma. Ethyl-β-apo-8′-carotenoate was used as internal standard. A Hypersil BDS RP-C_{18} column (150 mm × 4.6 mm), 5 μm particle size, was equilibrated with a mobile phase composed of acetonitrile and methanol (50:50, v/v). Its flow rate was 1.5 ml/min. The elution time for lycopene and ethyl-β-apo-8′-carotenoate was approximately 11 and 5 min, respectively. Calibration curves of lycopene were linear in the concentration range of 3–200 ng/ml in plasma. Limits of detection and quantification in plasma were 1 and 4 ng/ml, respectively. Recovery was greater than 97%. Intra- and inter-day relative standard deviation for lycopene in plasma was less than 1.8 and 3.1%, respectively. This method was applied to the determination of lycopene plasma levels after single dose administration to dogs.

Keywords: Lycopene; RP-HPLC; Canine plasma; Liquid–liquid extraction (LLE)

1. Introduction

Lycopene, an acyclic non-provitamin A carotenoid (Fig. 1A), is responsible for the red color of tomatoes, guava, watermelon and pink grapefruit. The extended system of eleven conjugated and two non-conjugated double bonds makes this molecule the most efficient singlet oxygen quencher among thousands of natural carotenoids [1]. This red pigment may act as an antioxidant by quenching free radicals formed during normal metabolism and may deactivate DNA chain-breaking agents that are implicated in some cancers [2]. Interest in lycopene research has been growing rapidly since several epidemiologic studies have shown correlation between diets rich in lycopene-containing foods and protection against various types of cancer [3–5]. Recently, epidemiologic and clinical studies indicated an association between tomato-rich diet and a lower risk of prostate cancer [3,5,6].

Lycopene has been determined in biological samples mostly by high performance liquid chromatography (HPLC). However, the majority of the existing chromatographic methods are referred to simultaneous determination of several antioxidants or carotenoids (including lycopene) [7–14], as well as their geometrical isomers in plasma samples [15,16]. Thus, these methods involve complicated instrumentation and time-consuming experimental procedures, including use of gradient elution or diode array detection. The growing importance of lycopene necessitates specific and reliable methods for its determination in plasma of experimental animals or humans. Such methods are very few [17–22] and some of them become complex because

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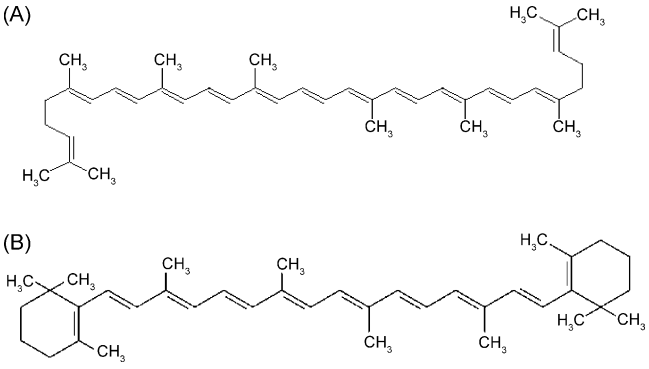

Fig. 1. Structures of lycopene (A) and ethyl-β-apo-8′-carotenoate (B).

they include analysis of lycopene geometrical isomers [17,19,20,22].

Our aim was the development of an optimized simple reversed phase HPLC method with visible spectrometric detection for the determination of lycopene in plasma samples. Since the dog is one of the most frequently used animal models for assessing oral absorption of dosage forms of xenobiotics [23], canine plasma can be additionally useful for three reasons. First, lycopene is a promising prostate cancer chemopreventive agent [5] and the dog is a good model for human prostate cancer [24]. The second reason relates to the fact that, unlike humans, dogs have no baseline lycopene blood levels [25] and thus, conclusions in regard to the absorption characteristics are easier to derive. Finally, the dog is one of the most frequently used models for assessing absorption of extremely lipophilic compounds via the lymph [26].

Advantages and novelty of the proposed isocratic method relatively to existing methods are either its simplicity in technique and instrumentation [17,19–22] or its simplified and optimized experimental procedures [18], e.g. the single liquid–liquid extraction step procedure, short elution time of lycopene, addition of a proper internal standard for increased accuracy and precision and lower limits of detection and quantification. Confirmation of the applicability of the developed method to pharmacokinetic studies of lycopene is also performed. Such studies have become imperative because of the dramatic increase of commercially available products (nutrition supplements) containing only lycopene in several formulations (tablets, soft gelatin capsules) and doses.

2. Experimental

2.1. Instrumentation

The chromatographic system used consisted of a Spectra System P1000 pump, a Spectra System UV 2000 absorbance detector extended to the visible region and an autosampler AS 3000. The above system was controlled by a Specta System Controller SN 4000 and a software package Chromquest (Thermoquest Inc., San Joe, USA). A Hettich centrifuge Universal 32R (Tuttlingen, Germany) was utilized to centrifuge plasma samples and a Techne Dri-Block® DB-3 (Cambridge, UK) sample concentrator for evaporation of samples.

2.2. Chemicals and reagents

All chemicals were of analytical purity grade. Methanol (MeOH), dichloromethane (CH_2Cl_2), hexane (C_6H_{14}) and acetonitrile (ACN) of HPLC grade were purchased from E. Merck (Darmstadt, Germany). All-trans lycopene was extracted and isolated from tomato paste (purity 93%, Laboratory of Pharmacognosy and Natural Products, National and Kapodistrian University of Athens, Athens, Greece). Pure all-trans-lycopene from tomato was purchased from Sigma–Aldrich, Inc. (St-Louis, USA). Ethyl-β-apo-8′-carotenoate ($C_{32}H_{44}O_2$, Sigma Chemicals Co., St-Louis, MO, USA) was used as internal standard (IS) and was of analytical purity grade. Canine blank plasma was obtained from dogs hosted at an animal facility that operates in our laboratory according to European Union regulations for the maintenance and experimentation on animals and which has been approved by the Veterinary Directorate of the Municipality of Athens. Water purified with Labconco water pro ps system (Kansas City, MO, USA) was used in all procedures. Aliquots of lycopene-free pooled canine plasma were used for preparation of spiked plasma standards. Butylated hydroxytoluene (BHT) of analytical purity grade (Sigma Chemicals Co., St-Louis, Missouri, USA) was used as preservative.

2.3. Chromatographic conditions

A reversed-phase Hypersil BDS-C_{18} column (150 mm × 4.6 mm, 5 μm particle size) equipped with a precolumn Hypersil BDS-C_{18} (10 mm × 4 mm, 5 μm particle size) was used. Mobile phase was composed of acetonitrile and methanol (50:50, v/v) and mobile phase was degassed for 10 min with helium at a degassing rate of 20 ml/min. Its flow-rate was 1.5 ml/min. Injection volume was 20 μl. Experiments were performed at ambient temperature. Absorption was measured at 472 nm, wavelength that was optimum for lycopene and satisfactory for IS. The elution time of lycopene and IS in plasma samples was approximately 11 and 5 min, respectively.

2.4. Solution preparation

2.4.1. Stock solutions

Stock solution of lycopene (50 μg/ml) was prepared by dissolving 5 mg of this compound in 100 ml of a mixture of CH_2Cl_2–C_6H_{14} 25:75 (v/v). Stock solution of IS (50 μg/ml) was prepared by dissolving 5 mg of ethyl-β-apo-8′-carotenoate in 100 ml of a mixture of CH_2Cl_2–C_6H_{14} 25:75 (v/v). Stock solutions were stored at −20 °C and were stable for at least 1 month.

2.4.2. Standard solutions

Lycopene working standard solutions were prepared in the concentration range of 3–200 ng/ml for establishment of the linearity range, construction of calibration curves and evaluation of the precision in measurements of standards and estimation of limits of detection and quantification. All dilutions to volume were performed with absolute ethanol. Calibration curves were performed either with solutions of standards in mobile phase or with spiked plasma standards. Concentration of the working solution of IS was 10 μg/ml. The analysis was carried out in the absence of direct sunlight.

2.5. Sample preparation

2.5.1. Analysis of plasma samples

One milliliter of plasma sample was transferred to a centrifuge tube and 1 ml of ethanol was added. After vortexing for 1 min, 35 μl of working solution of IS (10 μg/ml) were added to the previous solution and the mixture was vortexed for 30 s. Then, 2 ml of CH_2Cl_2 containing 0.1 mg/ml BHT, were added and the new mixture was vortexed for 1 min. Afterwards, tubes were centrifuged for 10 min in 4000 rpm ($1828 \times g$) at 10 °C. The clear organic phase was evaporated to dryness under nitrogen stream at room temperature. The residue was redissolved in 100 μl of CH_2Cl_2 and vortexed for 1 min. Then, 100 μl of mobile phase were added and the solution was vortexed for 1 min. Finally, part of the last solution was injected into the HPLC system.

2.5.2. Calibration curves

One milliliter of blank (lycopene-free) canine plasma was transferred to a centrifuge tube and 1 ml of lycopene working solution was added to it. Afterwards, the procedure described above in Section 2.5.1 was followed with vortexing for 1 min.

2.6. Data analysis

Calibration curves for lycopene were constructed, using peak-area ratios of lycopene standards to IS. Regression equations were obtained through unweighted least square linear regression analysis, applied to peak-area ratios as a function of lycopene concentration.

2.7. Canine in vivo data

A single dose oral administration of lycopene was performed in female, 4-year-old mongrel dog weighing 31 kg. Before administration, the dog was fasted for 16 h from food but not water. The dog was administered one soft gelatin capsule containing 10 mg of lycopene (Herbal Select, Ont., Canada) with 500 ml of milk (3.5% fat) via an orogastric tube. Blood samples were drawn by means of an indwelling catheter positioned in a suitable foreleg vein. After centrifugation, plasma was stored at −20 °C until assayed. This protocol had been approved by the Committee for Research of the University of Athens.

3. Results and discussion

3.1. Mobile phase

In this work, great effort was made to eliminate dichloromethane from the mobile phase. This solvent has been used by most researchers in lycopene analyses [10,18,21,23] to decrease lycopene elution time. However, dichlomethane is immiscible with water, and, therefore, very careful washing of the system to avoid clogging is required, when switching from such non-aqueous to other aqueous mobile phases. Moreover, dichloromethane is corrosive to certain parts of a HPLC system and leads to fast deterioration of its performance. In contrast, the mobile phase proposed in this study (methanol:acetonitrile 50:50, v/v) lacks the above disadvantages, whereas the elution time of lycopene was approximately 11 min. Such time is quite reasonable for any analysis and particularly of plasma samples.

3.2. Choice of internal standard

Several substances were tested as internal standards. Among these, ethyl-β-apo-8′-carotenoate (Fig. 1B) met all the typical requirements of a compound to be used as IS; its elution time was shorter than that of lycopene and its peak did not interfere with the matrix of plasma samples. Thus, this compound was chosen as the most appropriate in the present analysis.

3.3. Treatment of samples

Absolute ethanol was used for protein precipitation and liquid–liquid extraction for isolation of lycopene from plasma samples. To avoid a two-step extraction procedure with hexane that most researchers followed [18,20,21], dichloromethane was used instead. A single step liquid–liquid extraction with hexane was not sufficient as several researchers have pointed out [27,28].

Dichloromethane was chosen because lycopene was extracted quantitatively with a single-step extraction, while other plasma components were not. Optimization of the volume of dichloromethane was also performed; 2 ml of dichloromethane was the optimum volume for lycopene to be extracted from 1 ml of plasma. Higher dichloromethane volumes unreasonably increase the time of vaporization of samples. The extraction and reconstitution steps were very crucial because not only quantitative isolation of lycopene was accomplished but also preconcentration of injected solutions (five times) was achieved.

3.4. Selectivity

Typical chromatograms of blank plasma and a spiked plasma standard, obtained under the optimized experimental conditions, are shown in Fig. 2A and B, respectively. Good

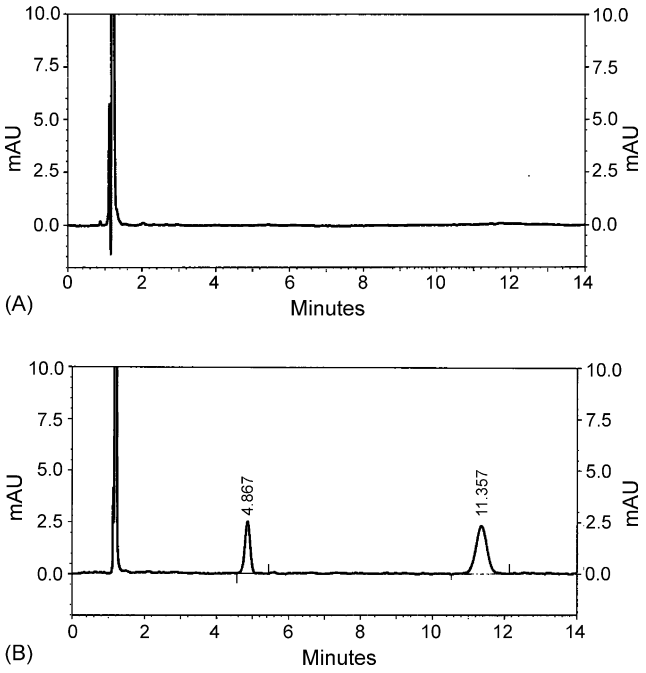

Fig. 2. Typical chromatograms of blank plasma (A) and a spiked plasma standard (30 ng/ml) (B). Retention times for IS and lycopene were 4.87 and 11.36 min, respectively. The chromatographic conditions used were: BDS RP-C_{18} column (150 mm × 4.6 mm), mobile phase acetonitrile–methanol (50:50, v/v), flow rate 1.5 ml/min, detection wavelength 472 nm and room temperature.

resolution for every peak and its nearest ones was assured by R_s values, which were greater than 5. Also, both lycopene and IS peaks were symmetrical (asymmetry factors were between 1.05 and 1.10).

3.5. Calibration curves

Linear calibration curves for lycopene were obtained throughout the concentration range studied. Regression analysis was performed for the ratios of peak-areas of lycopene to that of the IS (y), versus lycopene concentration (x). The results are tabulated in Table 1. In each case, the slope of the calibration curve obtained from standard solutions prepared in plasma matrix was not statistically different from that obtained in mobile phase (t-test, 95% confidence interval).

3.6. Precision and accuracy

To examine precision of the proposed HPLC method, precision of standards and samples was obtained. After preparing and measuring standards of the same concentration of lycopene in triplicate, values of intra- and inter-day relative standard deviation (R.S.D.) were calculated. Results showed that intra-day relative standard deviation for spiked lycopene in plasma was less than 1.8%, while the corresponding inter-day value was less than 3.1%.

The robustness of the proposed method was assessed with respect to small alterations in several experimental parameters which were slightly different from day to day. Deliberate changes in mobile phase from ACN/MeOH (50:50, v/v) to (48:52, v/v) and (52:48, v/v) did not change the results for lycopene more than 1.6%. During these changes, the parameters t_R, a and R_s remained statistically the same, probably because the present method is a non-aqueous reversed-phase HPLC approach. Similar observations were made, changing the flow rate of mobile phase from 1.5 ml/min to 1.6 and 1.4 ml/min, the volume of dichloromethane used for liquid–liquid extraction from 2.0 to 1.9 ml and 2.1 ml and the centrifugation time from 10 min to 9 and 11 min. All these changes did not alter the resulting lycopene concentration more than 3.5%. This figure is within the R.S.D. range of the method.

Ruggedness of the developed method was indicated by the between days precision because it included changes in reagents, chemicals and solvents. Moreover, using three different columns of the same company (Hypersil BDS RP-C_{18}), the parameters t_R, a and R_s of the chromatographic peaks remained statistically the same. At the same time, in the determination of lycopene changes of the results up to 3% were noticed.

Use of internal standard solution in analyses of biological samples assures ruggedness and robustness of this method. Indeed, in the determination of lycopene in plasma samples, the use of IS eliminated variations that are unavoidable during a liquid–liquid extraction procedure.

Accuracy of the developed method was examined by extensive recovery studies at low, medium and high concentration values. These results are summarized in Table 2. Recovery data were determined as ratios of integrated peak areas of lycopene to internal standard in standard solutions prepared in plasma matrix compared to the working standards in mo-

Table 1
Analytical parameters of calibration curves of lycopene in mobile phase and canine plasma in the concentration range of 15–1000 ng/ml of injected solution

Matrix	Regression equation[a]		
	Intercept, $a \pm$ S.D. ($\times 10^5$)	Slope, $b \pm$ S.D. ($\times 10^3$)	Correlation coefficient[b], r
Mobile phase	12 ± 14	202.3 ± 7.3	0.997
Plasma	7.6 ± 9.9	207 ± 10	0.995

The corresponding range in canine plasma was 3–200 ng/ml, considering the five times preconcentration.

[a] Linear unweighted regression analysis, with a regression equation $y = a + bx$, where y was the peak-area ratio of lycopene to IS and x was lycopene concentration in ng/ml. S.D. is the standard deviation of intercept and slope.

[b] The number of points in each calibration curve was 7 and each point was the mean of three experimental measurements.

Table 2
Recovery data for the determination of lycopene in canine plasma

Concentration (ng/ml)		Mean % recovery ± S.D.[a]
Canine plasma	Injected	
6	30	97.9 ± 3.6
14	70	99.2 ± 1.5
40	200	99.82 ± 0.54
100	500	100.8 ± 1.1
200	1000	99.56 ± 0.78

[a] S.D. is the standard deviation of the mean % recovery; standard solutions were prepared and measured in triplicate.

bile phase. An estimation of the recovery of the method can also be obtained comparing the slopes of calibration curves in mobile phase and in plasma matrix.

3.7. Limits of detection (LOD) and quantification (LOQ)

LOD was defined as the analyte concentration that gives a signal equal to $y_b + 3.3s_b$, where y_b is the signal of the blank and s_b is its standard deviation. Similarly, LOQ was defined as $y_b + 10s_b$. In an unweighted least-squares method is quite suitable in practice to use the statistic $s_{y/x}$ [29] instead of s_b and the value of the calculated intercept a instead of y_b. Values of $s_{y/x}$ and b were calculated from calibration curves of plasma lycopene standards obtained in a very low concentration region in plasma (3–40 ng/ml). Thus,

$$\text{LOD} = \frac{3.3s_{y/x}}{b} \quad \text{and} \quad \text{LOQ} = \frac{10s_{y/x}}{b}$$

where b is the slope of the regression line.

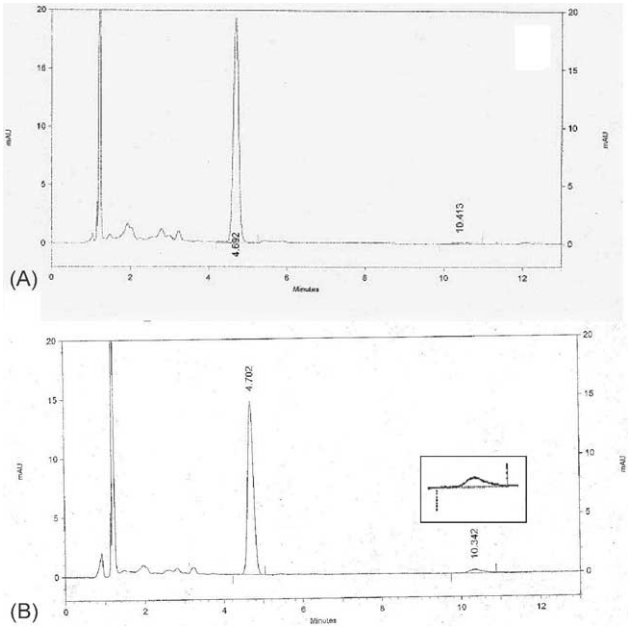

Fig. 3. Chromatograms of canine plasma (A) prior to lycopene administration and (B) 2 h after a single administration of a soft gelatin capsule of lycopene (10 mg), where lycopene plasma concentration (3.6 ng/ml) was close to the LOQ of the method.

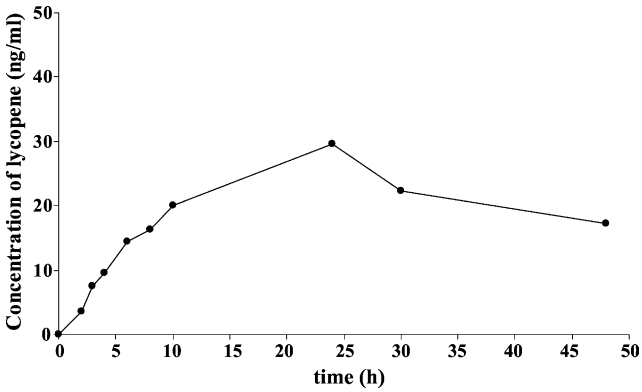

Fig. 4. A pharmacokinetic profile of lycopene in canine plasma after a single administration of a soft gelatin capsule of lycopene (10 mg).

Based on the above equations, the calculated LOD value was 1 ng/ml while the LOQ was 4 ng/ml in plasma. A chromatogram of dog plasma, following lycopene administration, close to the LOQ of the method along with the chromatogram of the blank sample of the same pharmacokinetic profile are presented in Fig. 3.

3.8. Canine in vivo data

Fig. 4 shows a profile of lycopene concentration in plasma after administration of a soft gelatin capsule to a dog. It is evident that commercially available oral formulations contain low doses of lycopene leading to low plasma levels after single administration. The long absorption and elimination phases have been also reported by others [25].

4. Conclusion

A simple, fast and reliable reversed-phase isocratic HPLC method with visible spectrometric detection for the determination of lycopene in canine plasma has been optimized and validated. Advantages of the proposed method include: a simplified single-step liquid–liquid extraction procedure (better recovery and shorter sample preparation periods), use of IS (improved precision and accuracy) and low quantification limits (due to the preconcentration of injected solutions) allowing application of this method to single dose of lycopene pharmacokinetic studies in dogs.

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