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Development of a Rapid HPLC-UV Method for Analysis of Menaquinone-7 in Soy Nutraceutical

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Abstract

Among all fermented foods, fermented soybeans (soy nutraceutical) were found wide applications as nutraceutical due to nutritional benefit. The vitamin K2, menaquinone-7 (MK-7) is commonly used for preventing osteoporosis and is an important secondary metabolite of soy nutraceutical. A consistent and improved high-performance liquid chromatography (HPLC) method for determination of MK-7 in fermented soybean is developed. The analysis was achieved on Lichrospher-100, RP-C_{18} (5 µm) column with a dimension 125 mm × 4.0 mm, with detection at 248 nm using a gradient mobile phase mixture of water and methanol (1:1 v/v) acidified to pH 3.0 by orthophosphoric acid and acetonitrile with a flow rate of 1.2 mL min^{-1}. Under these conditions, the analysis of MK-7 was achieved in less than 4 min. The retention time was found to be 2.38 min. The calibration curve for MK-7 was linear in the range of 2.5-20 µg mL^{-1} with R^2=0.9997. The proposed method was successfully employed for quantification of the MK-7 present in soy nutraceutical.

Keywords: Menaquinone-7; Vitamin K2; Fermented soybean; HPLC-UV; *Bacillus subtilis*

Introduction

Menaquinone-7 (MK-7) is a vitamin K-2 analogue and plays an important role in the carboxylation of γ-glutamate residues of the osteocalcin [1-4]. The γ-carboxylated osteocalcin promotes the mineralisation of bone in osteoblasts in bone metabolism, thereby help in the prevention of osteoporosis [5,6]. High dietary MK-7 intake reduces coronary calcification and prevents cardiovascular disease [7]. The important source for MK-7 includes fermented soybeans like natto and found both in animal products and in the intestine [8,9]. There are reports on the biosynthesis of MK-7 from vitamin K1 by microorganisms [10]. Due to anti-osteoporosis activity, MK-7 incorporated into the multivitamin formulation and along with calcium and vitamin D. Therefore, analysis of MK 7 is required and urgently needed.

The chromatographic analysis of MK-7 will play an important role in establishing the quality of MK-7 containing fermented food, pharmaceuticals and its metabolism/biosynthesis. The objective of this study was to develop and validate a rapid HPLC-UV method and to investigate the concentration of the MK-7 present in nutraceutical produced under solid-state fermentation of soybean by *Bacillus subtilis* fermented soybeans by a newly developed high-performance liquid chromatography-UV method.

Materials and Methods

Materials and microorganism

The culture of *Bacillus subtilis* NCIM 2708 was obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, Maharashtra, India. It was maintained on slants of the nutrient agar mediums at 4°C and sub cultured at every 30 days interval. All the chemicals and solvents used in the research procured from Merck, Mumbai, India. Microbiological media procured from Hi-Media, Mumbai, India. Soybean verity SL-525 and DS-9814, collected from the Pulse Laboratory of Indian Agricultural Research Institute, New Delhi, India. The reference compound MK-7 obtained from Medley Pharmaceuticals, India.

Preparation of seed culture

Microbial suspension of *Bacillus subtilis* NCIM 2708 was prepared from actively growing slants in distilled sterile water. Microbial suspension was inoculated into the seed culture medium (1% v/v) containing soybean powder 6% (soybean variety, SL-525), sodium chloride 0.5% and distilled water adjusted to pH 7.0 with 0.1 N HCl or 0.1 N NaOH [11] and incubated at 37°C for 24 h in a shaker incubator at 180 rpm.

Soy nutraceutical production

Soybean based nutraceutical was prepared by the solid-state fermentation of soybean seeds by *Bacillus subtilis* NCIM 2708. Soybean seeds (variety DS-9814) were prepared for the fermentative production of MK-7 by successive procedures of washing, soaking, boiling and dehulling respectively. The prepared soybean seeds were sterilised and seed culture of B. subtilis NCIM 2708 was added at a concentration of 1 mL g^{-1}. Fermented in a humidity incubator at 37°C and 75% relative humidity for 24 h.

After solid-state fermentation, the fermented soybean seeds are kept at 4°C for 7 days in order to achieve the ageing process [12]. Aged fermented soybeans were autoclaved and extraction of MK-7 was carried out with different solvents.

Extraction of MK-7 from fermented soybeans

Fermented soybean seeds DS-9814 (5 g) were triturated by the mortar-pestle. To the fermented soybean DS-9814, 15 mL of different non-polar and medium polar extracting solvents i.e., propan-2-ol & n-hexane (1:2 v/v), toluene, acetonitrile and ethanol were added and were mixed by vigorous shaking for 10 min. The mixture was centrifuged at 3000 rpm for 5 min and the organic layer was separated and concentrated up to 1 mL. Samples obtained were filtered through 0.45 µm membrane filter and were analysed by HPLC for quantification of MK-7.

Preparation of standard MK-7

Different concentrations ((2.5, 5.0, 10, 20) µg mL^{-1}) of standard MK-7 were prepared in a solvent mixture of water & acetonitrile (2:8 v/v). Standard solutions were filtered through a 0.45 µm membrane filter and were analysed by the HPLC to prepare the standard plot of MK-7.

Chromatographic condition and analysis of MK-7

The extracted and standard MK-7 were analysed by quaternary HPLC system (Shimadzu Japan). The system software was class-VP equipped with Lichrospher-100, RP-C_{18} column with 5 µm sizes and a dimension of 125 mm × 4.0 mm. The column temperature was kept at 25°C. Elution of MK-7 was optimised by using different mobile phase with different flow rate under isocratic and gradient mode conditions. Detection of MK-7 was carried out by UV detector. Peaks were analysed by using software Class VP (Shimadzu, Japan). Some of the close chromatographic conditions under which the MK-7 was detected are presented below.

Chromatographic condition I

A mixture of water and methanol (1:1 v/v) acidified to pH 3.0 by orthophosphoric acid (A) and acetonitrile (B) with a flow rate of 1.2 mL min^{-1} under gradient mode was used as mobile phase. The absorbance of MK-7 was detected at 248 nm with a gradient elution (Table 1).

Events Number	Time	Solvent	%
1	0.01	Acetonitrile	80
2	3.50	Acetonitrile	80
3	4.50	Acetonitrile	100
4	6.50	Acetonitrile	100
5	10.00	Acetonitrile	80

Table 1: The gradient time programme for chromatographic condition I.

Chromatographic condition II

Methanol (A) and Acetonitrile (B) (1:1 v/v) with a flow rate of 1 mL min^{-1} under isocratic condition was used as mobile phase with absorbance at 254 nm.

Chromatographic condition III

Methanol (A) and water (B) (95:5 v/v) with a flow rate of 1 mL min^{-1} under the isocratic mode of elution with detection of MK-7 at 254 nm.

Results and Discussions

Chromatographic analysis of standard MK-7

The standard MK-7 concentration of 10 µg mL^{-1} eluted with different chromatographically conditions shows different R_t and percentage elution pattern (Figures 1a-1c). Under chromatographic conditions, I the R_t was found to be the lowest i.e., 2.3 min with 99% elution. Under the chromatographically condition, II the R_t was found to be the highest i.e., 4.8 min with 68% elution. Under the chromatographically condition, III the R_t was found to be 3.4 min with 90% elution. From results, it shows that gradient mode of elution resulted enhanced MK-7 elution than an isocratic mode of elution. Further, under gradient mode, the elution time was decreased considerably in comparison to the isocratic mode. Out of chromatographically condition, I and III, condition I was better than condition III for analysis of MK-7.

Linearity of analysis

Different dilution of MK-7 ((2.5, 5, 10, 20) µg mL^{-1}) was prepared and analysed by the chromatographic condition, I. The solutions were injected in triplicate, and the regression equation was found Y (MK 7)=118202 × +22026 (R^2=0.9997) by plotting the peak area (Y) versus the MK-7 concentration (X) expressed in µg mL^{-1}. The coefficient (R^2) obtained for the regression line demonstrates the excellent relationship between peak area and the concentration of MK-7. The chromatograms were represented in Figure 2.

Precision of analysis of HPL chromatogram method

The precision of the chromatographic analysis of MK-7 was observed in the form of percent relative standard deviation (% RSD) and was estimated by measuring the repeatability of intra-day analysis on five replicate of the MK-7 solution at the highest concentration. The RSD value for peak area and retention time (R_t, min) was found to be 0.16 and 0.14 respectively.

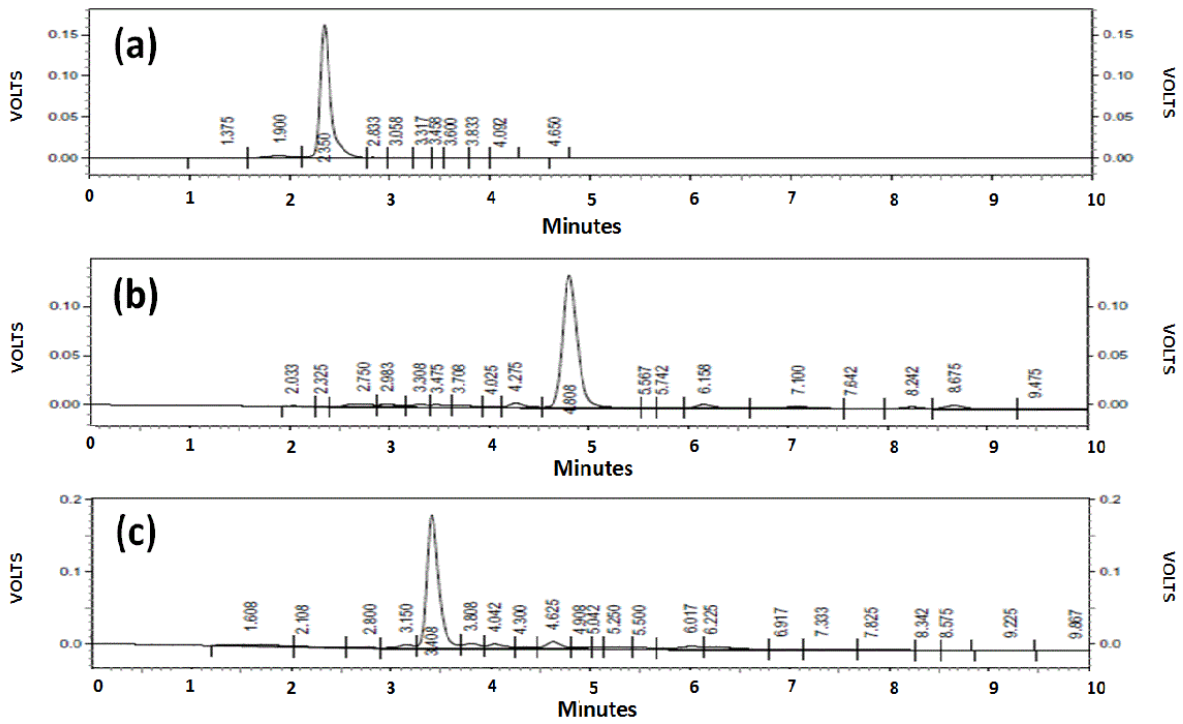

Figure 1: The chromatograms of MK-7 (10 μgmL^{-1}) eluted under chromatographic conditions I (a), chromatographic conditions II (b) and chromatographic conditions III (c)

Limits of detection and quantitation of MK-7 analysis

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) of MK-7 analysis were determined by the calibration plot method [13]. LOD and LOQ were calculated by use of the equations:

$$LOD = C_{lod} \times R_{vr}/S_l$$

&

$$LOQ = C_{loq} \times R_{vr}/S_l$$

Where C_{lod} and C_{loq} are the coefficients for LOD and LOQ, Rvr is the residual variance of the regression, and S_l is the slope. Calculations were performed by using values of C_{lod} and C_{loq} of 3.3 and 10. LOD and LOQ of MK-7 were found to be 0.49 μg mL^{-1} and 1.499 μg mL^{-1} respectively.

Accuracy and specificity of the HPL chromatogram analysis

Accuracy and specificity of the chromatographic method was tested on fermented soybean seeds containing MK-7. The analysis was carried out with and without co-injection of MK-7. The MK-7 extraction from nutraceutical was carried out by successive extraction through propan-2-ol and n-hexane (1:2 v/v), toluene, acetonitrile and ethanol separately. Propan-2-ol and n-hexane extract found to be having maximum, 8.28 μg of MK-7 per gram while acetonitrile extract found to contain 3.24 μg of MK-7 per gram of soy nutraceutical. However, MK-7 was undetected toluene and ethanol extract [8].

The retention time for MK-7, extracted from fermented soybean seeds was increased to 2.44 min from 2.39 min. The accuracy & specificity of the analysis and extraction procedure was carried out by adding internal standard of MK-7 (2.5 μg mL^{-1}) to one gram of fermented soybeans and extracted with propan-2-ol and n-hexane (1:2 v/v) shows R_t at 2.35 min and concentration was found to be 11.77 μg g^{-1} with 89.32 % elution. The chromatograms of extracted MK-7 from soybean nutraceutical with and without internal standard were shown in Figure 3. In a study determination of MK-7 by HPLC was obtained by using mobile phase with a mixture of methanol and ethanol 95:5 (v/v).

The fluorescence detector was used for the analysis of MK-7 with a flow rate of 1 mL min-1 and the retention time was 3.0 min [14]. In the present study of the analysis of MK-7 through HPLC by gradient elution program, the mobile phase was a mixture of water (pH adjusted to 3.0 with orthophosphoric acid) and methanol (1:1 v/v) and acetonitrile in a ratio of 2:8 (v/v) with a flow rate of 1.2 mL min^{-1} by using UV detector at a λ_{max} of 248 nm. The benefit of this modification was that total retention time of MK-7 was decreased from 3 min to a retention time of 2.3 min and analysed by UV detector, which is comparatively cheaper and widely used than a fluorescence detector.

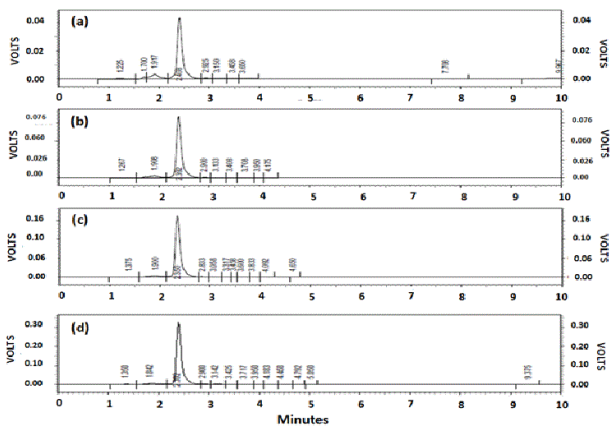

Figure 2: The chromatograms of MK-7 of concentration 2.5 µg mL^{-1} (a), 5 µg mL^{-1} (b), 10 µg mL^{-1} (c) and 20 µg mL^{-1} (d) eluted under chromatographic conditions I.

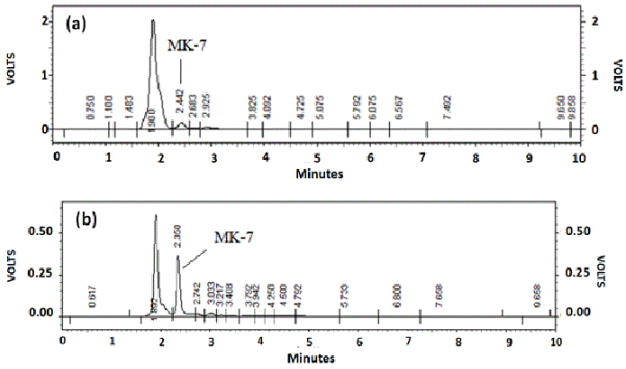

Figure 3: The chromatograms of extracted MK-7 from soybean nutraceutical by propan-2-ol and n-hexane (1:2 v/v) solvents without (a) and with internal standard (b) of concentration 2.5 µg mL^{-1}.

Conclusion

The newly developed gradient HPLC-UV method for analysis MK-7 in soybean nutraceutical is specific, accurate, rapid and precise. The HPLC of MK-7 achieved by UV detector under the gradient mode of elution. The peak area and MK-7 concentration show excellent correlation. Further, the highest amount of MK-7 was extracted with using a solvent mixture of propan-2-ol and n-hexane (1:2 v/v) from fermented soybean. The developed method can be useful for extracting and analysing the MK-7 present in fermented soybean seeds and multivitamin formulation.

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