CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.1343905

Available online at: http://www.iajps.com Research Article

VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TECHNIQUE FOR THE INVESTIGATION OF THYMOL AND CARVACROL IN *THYMUS SERPYLLUM* L. INDIGENOUS TO BALOCHISTAN

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Abstract:

Thymus serpyllum L. belonging to the family Lamiaceae, is a popular therapeutic plant which contains ingredients such as thymol and carvacrol which has been generally utilized in various pharmaceutical products . Thymol and carvacrol are mostly analyzed by gas chromatography (GC) method however, in this project validation of a high performance liquid chromatography (HPLC) technique used and developed for the quantification of thymol and carvacrol in Thymus serpyllum L. Thymus oil of the herbal plant was quantified by the GC and HPLC methods. The HPLC method selected with Hypersil ODS C 8 (250 X 4.6mm) column and an isocratic acetonitrile: water: orthophosphoric acid (60:38:2) used as mobile phase which was set at a flow rate of 1.5 ml/minute. The analytical method was used for linearity, selectivity (r^2>0.997), for thymol and carvacrol The limit of detection (LODs) and limit of quantification (LOQs) were analyzed to be 0.015, 0.050 µg/ml and 0.5 , 1.9 µg/ml for thymol and carvacrol respectively. The precision (inter day 3.3-3.6, 3.9-4.3) and (intraday 1.1-1.8, 1.8-2.8) and recovery (98.1%, 97.2%) for thymol and carvacrol respectively. The quantity of thymol and carvacrol in thymus oil observed by HPLC (8.3%, 3.03%) and GC (7.7%, 2.95 %) were observed by statistical analysis and they found good result.

Keywords: *Carvacrol, HPLC, GC, Thymus serpyllum L., method Validation.*

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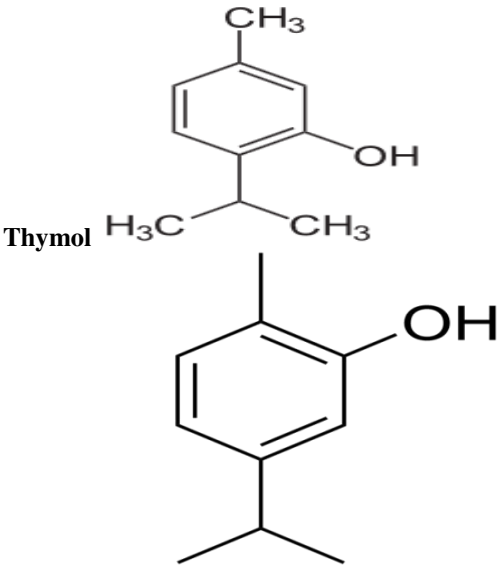
Please cite this article in press Rahman Gul et al., ***Validation of a High Performance Liquid Chromatography Technique for the Investigation of Thymol and Carvacrol in Thymus Serpyllum l. Indigenous to balochistan.,*** *Indo Am. J. P. Sci, 2018; 05(08).*

INTRODUCTION:

There are 300-400 species in the Genus Thymus, with a number of them utilized in Folk medicines. Traditionally used and the most important medicinally is *Thymus serpyllum* L. which is used for bronchitis, cough, laryngitis and gastritis [1,2,3]. The main chemical compound which is mainly present in *Thymus serpyllum* L. is thymol and carvacrol shown in (Fig. 1). Thymol quantity is much higher in thymus oil than the other phenolic components. This compound is 30 times more antioxidant and less toxic than other components [4,5]. Since these compounds are considered to be the active constituents of essential oil, many pharmaceutical products are standardized with thymol as active constituents. Therefore, A Validated method must be considered for the investigation of these constituents GC is the main and famous technique for the investigation of essential oil constituents in herbal plants [1,6,7,8]. However, it is not easy to find out essential oil in Pharmaceutical formulation directly. Hence, usages of other methods are complicated for the quantification of essential oil [9,10,11]. HPLC is one of the advance and reliable technique for the analysis of herbal plant constituents of non-volatile and volatile components [12,13]. In the current study a reliable Validated HPLC technique used for the quantification of thymol and carvacrol in *Thymus serpyllum* L., essential oil has been injected and quantity of the two components in the essential oil of the herbal plants which were quantified by the novel reliable HPLC method compared to those investigated by GC technique.



Thymol

Carvacrol

***Fig. 1*:** Structure of Thymol and Carvacrol

MATERIALS AND METHODS

Chemicals

Thymol and carvacrol were chosen as the analytical standard for *Thymus serpyllum* L. was gotten from Merck Serno Quetta, Factory, Pakistan. All the other solvents and chemical used were of Merck (Darmstadt, Germany)**.**

Plant Material

Thymus serpyllum L. Ariel parts were collected in May 2016 from Ziarat (Balochistan, Pakistan) and Identified by Dr. Atta Muhammad Kakar, Botany Department, University of Balochistan, Quetta, Pakistan.

Extraction of essential oil

Dried plant material from *T. Serpyllum L.* was hydro distilled for 3 hr in a Clevenger type equipment according to the European Pharmacopoeia. The oil obtained was consequently dried over anhydrous Na_2SO_4 and kept at 4ºC until investigation.

UV-Spectrum

10mg of Thymol was weighed correctly and transferred to a 100 ml glass volumetric flask. The drug was dissolved in methanol and the volume made to 100 ml with methanol. further dilutions 1mg / ml were prepared with acetonitrile, water, orthphosphoric acid in ratio (60:38:2). Resulting solutions were scanned in the range of 400 to 200 nm as shown in (Fig. 2). Using UV-Visible Spectrophotometer .(UV-1600 Shimadzu)and λ_{max} was determined

Instrumentation

HPLC technique was performed using System (Agilent Technologies, 1100 Series, USA with LC-10 AT VP pump). Equipped with, DGU-AM 14 degasser, manual injector system, SPD-10 AVP UV–VIS detector, and Hypersil ODS C 8 (250 X 4.6mm) column. Agilent software 1100 Series, USA Chem Station Series 2001-2005 was used for data collection and processing. The chromatographic conditions used for analysis were as follows: the mobile phase composed of (Acetonitrile: water: orthophosphoric acid) at ratios of 150:850:2.respectively, The mobile flow rate was 1 mL/min, and injected volume was 20 µL, samples were detected by an ultraviolet-visible detector at a wavelength of 274 nm .The GC used was Shimadzu with a capillary column CP-SIL8 (60 m x 0.25 mm i.d., 0.22 um f.t), the carrier gas was He at flow rate 1mL/minute .A Flame detector was utilized. The Temperature of the Column was 60ºC for 1 min than increase up to 230ºC at a rate of 2

^{o}C/min and end time was 30 min.

Thymol and carvacrol Quantization by HPLC.

Sample preparation

10 mg of thymus oil was transferred to a glass volumetric flask and the samples were diluted to 100 mL with acetonitrile, water (70:30) (100 µg/mL), 6 samples were arranged and each sample was injected in triplicate.

Standard Preparation

A stock solution of thymol (2 mg/mL), and carvacrol (0.2 mg/mL), were arranged in acetonitrile, water (70:30) different concentration (0.3125-5 µg/mL for thymol and 2-10 µg/mL carvacrol) were prepared from already prepared stock solutions to plot the calibration curve of thymol and carvacrol.

Validation

The consistency of new HPLC technique for investigation of thymol was recognized during Linearity, selectivity, precession and recovery.

Linearity

Linearity was evaluated through the relationship between thymol and carvacrol, absorbance produced by UV-HPLC detector. Coefficient (r^2) was determined by way of least squares analysis. The calibration row was drawn in two replicates of both thymol and carvacrol.

Limit of detection (LOD) and Limit of Quantization (LOQ)

Specificity

Injection of samples placebo in duplicate under the developed method of HPLC under the set conditions, showed no interference (Figs.3 and 4). Thus the peak of thymol and carvacrol were evaluated with the resolution from the adjacent eluting peak [13,14].(Shekarchi et al., 2010; ICH, 1996).

Precision

The precision in every technique shows the amount of distribution with the sequence of determination of the similar samples. Three samples of different concentration (80%, 100%, 120%) were investigated (intraday) the same day and the three samples for (Inter day) on consecutive day. The RSD % was calculated.

Accuracy

The same method was utilized for the analysis of known concentration of thymol and carvacrol with different concentrations 10 to 30 µg/mL of thymol and 1 to 3 µg/mL carvacrol and observed in the three determinations, recovery was statistically calculated in each sample. Each sample was injected in triplicate.

GC Method for the Analysis of Thymol and carvarol

Sample Preparation

100 mg of thymus oil was poured in to the glass volumetric flask and prepared dilution with 5 mL n hexane. 6 samples of thymus oil about 8% thymol and 3% Carvacrol were organized and each one was injected in triplicate to the GC apparatus.

Standard Preparation

Stock solution of reference thymol 20 mg/mL and 2 mg/mL of carvacrol were dissolved in hexane and sequential dilution of thymol 2-10 mg/mL and 0.2-1.0 mg/mL for carvacrol were prepared from the stock solution. The Isopropyl alcohol was used as internal standard

Statistical Data Analysis

The observed data were analyzed by SPSS software and the data were reported as mean ± SD.

RESULTS AND DISCUSSION:

The Wave length maximum Absorption (λ_{max}) was found to be 274nm. The reported value is also 274.0nm. This value was selected in the determination of Thymol and Carvacrol for the essential oil used in HPLC Technique

Gas chromatography is the general method used for the analysis of thymol and carvacrol in thymus oil, some researchers have discussed this method for the analysis of thymol and carvacrol [2,7,10]. But it has some limitation to be used for many complicated samples. Thus, this study has been utilized on the quantization and separation of thymol and carvacrol by reliable new validated HPLC technique in *Thymus serpyllum* L. thymus oil and the result of HPLC technique were compared with those of GC method quantitatively. carvacrol and thymol peaks were observed in retention time 5 and 6 as shown in Fig. 5

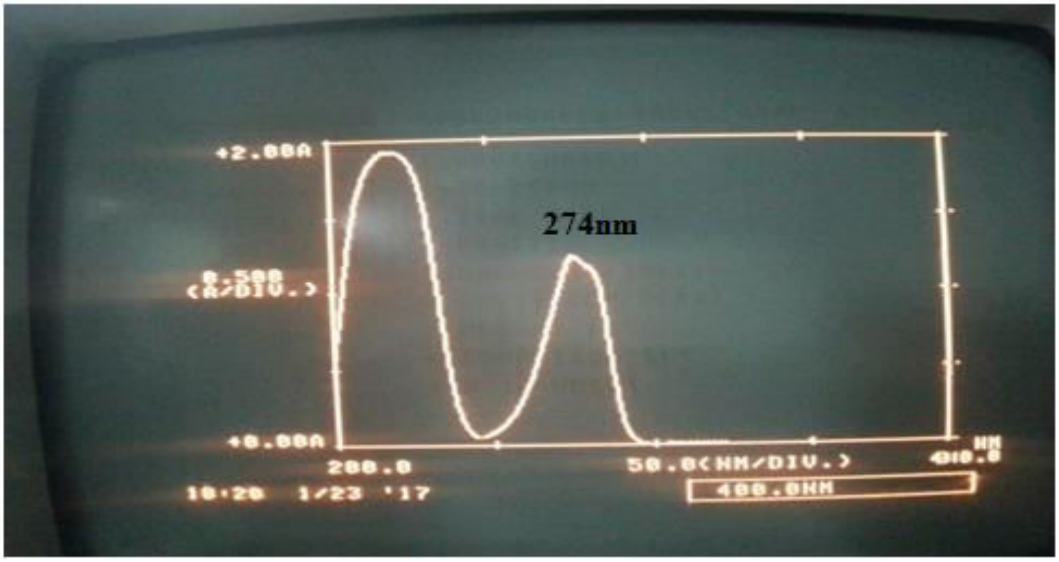


Fig. 2: UV absorption spectrum at 400-200 nm.

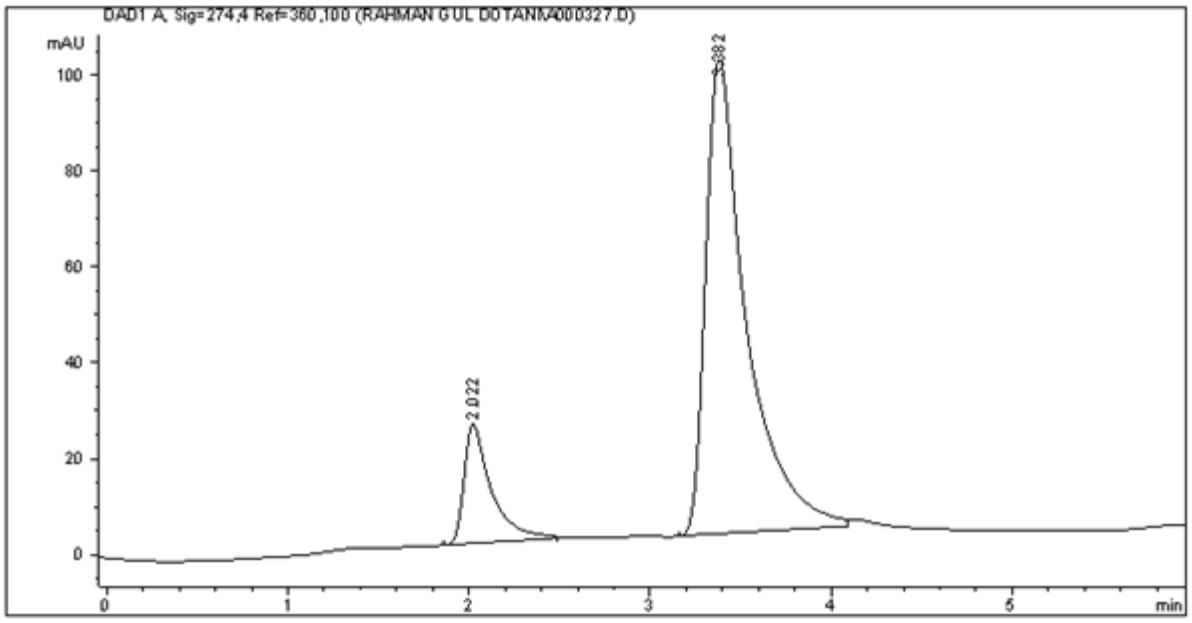


Fig. 3: HPLC Chromatogram of thymol standard.

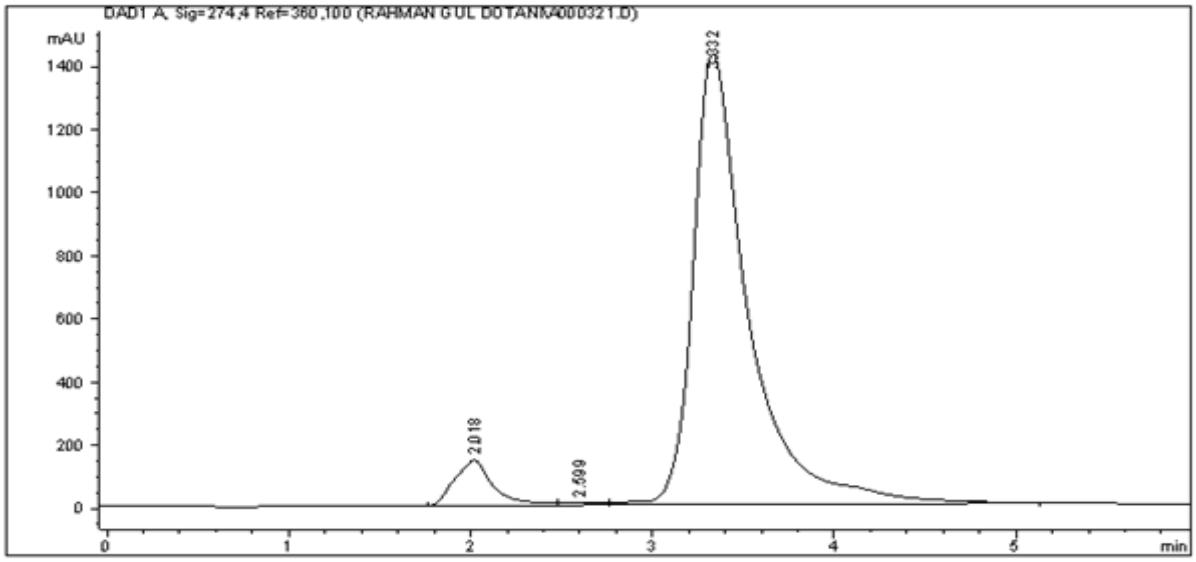


Fig. 4: HPLC Chromatogram of *Thymus serpyllum* L. essential oil.

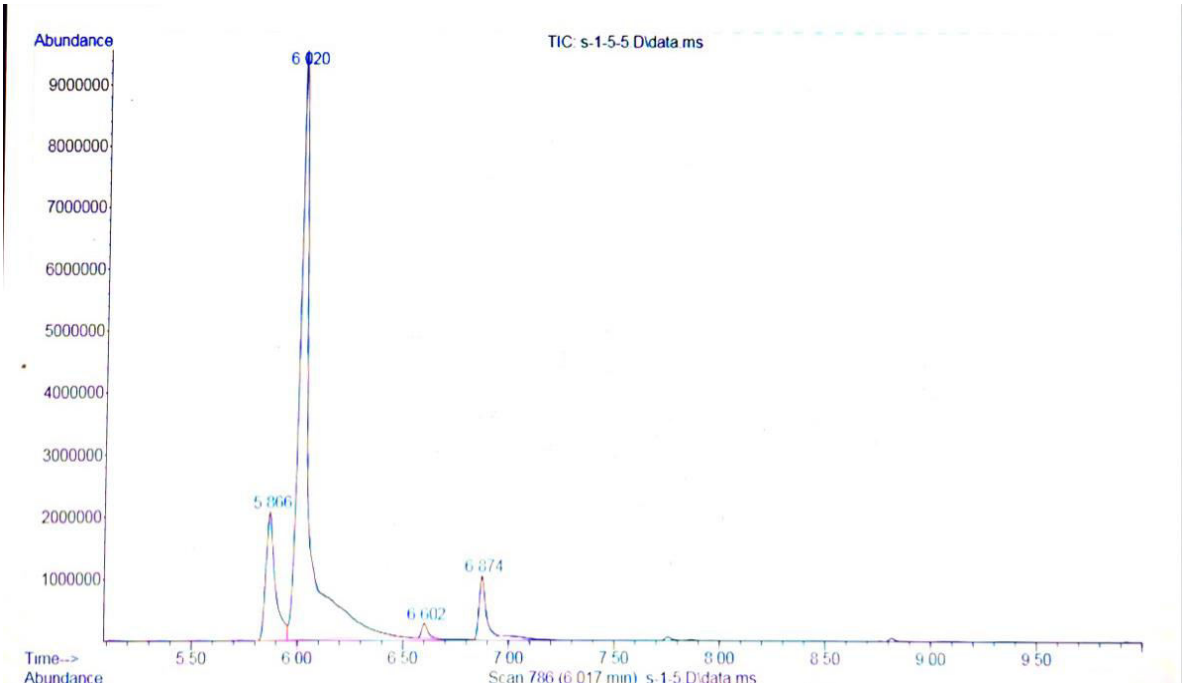


Fig. 5: GC chromatogram of *Thymus serpyllum* L. essential oil

The GC chromatogram of the essential oil were well resolved, separated. Quantitative results showed 7.7 % of thymol and 2.91% of carvacrol in *Thymus serpyllum* L essential oil Table 1.

The quantitative result of thymol and carvacrol in thymus herbal plants essential oil by HPLC technique as well resolved and showed good peak and separation as showed in (Figure 3 and 4) via HPLC technique the concentration of thymol in the thymus essential oil was assessed 8.2% and carvacrol 3.03%. The result obtained from HPLC and GC were very close between two methods

Table: 1, *Evaluation of HPLC and GC technique for the investigation of thymol and carvacrol in Thymus serpyllum*

L. thymus oil

Compound	Method	Concentration (%w/w)	Mean SD
Thymol	HPLC	8.9 7.8 8.1 8.2 8.3 7.8	8.2±0.3
	GC	7.3 8.6 7.6 6.9 7.9 7.9	7.7±0.5
Carvacrol	HPLC	3.2 3.0 3.1 2.2 3.3 3.4	3.03±0.2

Method Development

After several trial of mobile phase, acetonitrile, water and orthophosphoric acid in different ratios, the ratio 60:38:2 in an isocratic system was finally chosen for good resolution and separation. The flow rates were managed between 0.5 and 1.5 mL/min. The flow rate of 1 mL/min gave a maximum signal/noise ratio with a good resolution and separation time (5 minutes).Using different columns such as C_8 and C_{18} the best separation was obtained by C_8 column. The retention time for carvacrol and thymol were showed to be 2.01 to 3.03 as shown in Fig. 3 and 4 and UV range indicated maximum absorption at 274 nm; the thymol and carvacrol were observed at this given wavelength. Similarly investigation of thymol and carvacrol was assessed by ODS column with mobile phase; methanol; Water; acetic acid (60:40:2) The mobile phase and method was found to be reliable but not efficient and showed good separation [11].

Essential oil contains many ingredients which are dissolved in non- polar solvents. So to get the best resolution and separation in HPLC Technique, the study was done by Solinas and Gessa for investigating Thymol and carvacrol in Thymus species by HPLC, all samples were dissolved in methanol, which is not better than mobile phase of acetonitrile and water. which may be the reason of overlapping of peaks in the chromatograms of thymol and carvacrol in herbal plants essential oil [15].

Validation method

The results achieved from new method validation for thymol and carvacrol analysis as per procedure, Linearity, selectivity, accuracy and precision indicated that the selected method was reliable (Table 2). Best linearity was observed for thymol and carvacrol peak area between concentrations of 0.3125-5 µg/ml with r^2 = 0.998 and 2 - 10ug/ml of carvacrol with r^2 = 0.9978 thymol and carvacrol peaks of standard and sample were almost pure and similar spectrally. LOD and LOQ were calculated as 0.015, 0.05 µg/mL and 0.50 and 1.90 µg/ml respectively. Precision results of thymol and carvacrol indicated RSD % <1.8 and 2.8 for inter day and RSD % <3.6and 4.3 for intraday. That was a reasonable accuracy, the recovery of the two levels was found to be 98.1 and 97.6 % respectively Table 3.

Table: 2,*Validation parameters for the quantification of Thymol and carvacrol in Thymus serpyllum L. thymus oil by HPLC*

Parameters	Result of thymol	Result of carvacrol
LOD&LOQ(µg/ml)	0.015 and 0.050	0.5 and 1.9
Linearity(r^2)	0.998	0.9978
Range (µg/ml)	0.3125-5	2-10
Intraday Precession 80,100,120% (RSD%)	1.1,1.2,1.8	1.9,1.8,2.8
Interday Precession 80,100,120% (RSD%)	3.3,3.6,3.5	3.9,4.3,3.8

Table: 3,*Recovery assessment of thymol in Thymus serpyllum L. thymus oil by HPLC technique*

Compound	Concentration (µg / ml)	Added (µg / ml)	Found (µg / ml)	Recovery %	Mean recovery %
Thymol	8.0±0.3	10	18	98.2	98.1±0.1
		30	38	96.5	
Carvacrol	3.0± 0.06	1	3.1	98.2	97.2±1.2
		3	6	96.5	97.3

CONCLUSIONS:

This new method validated for the most active component in *Thymus serpyllum* L. essential oil thymol and carvacrol quantization which were selective, precise and accurate. It is the best method for the Quality control of *Thymus serpyllum* L. essential oil to assess thymol and carvacrol in Thymus formulations. Which may not be investigated by other method easily, this is one of the best substitute to the other methods for the analysis of the constituents in plants.

ACKNOWLEDGEMENT:

The Author is thankful to the Health Department Government of Balochistan for providing Study Leave, and Dean of Pharmacy Department, Islamia University of Bahawalpur for support to complete this research.

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