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Research Article

Validated High Performance liquid Chromatographic Method for Estimation of Thiocolchicoside, and its Application to *Ex-vivo* Permeation Studies for Transdermal Therapeutic Systems

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ABSTRACT

A simple, accurate and sensitive high performance liquid chromatography (HPLC) method for estimation of thiocolchicoside (THC) in skin permeated samples was developed and validated. The chromatographic separation was accomplished on a C 18 column (Inertsil ODS 3V C-18, 250 × 4.6 mm, 5 µ)using a mixture of Buffer: Methanol (45:55) as mobile phase. The flow rate was fixed at 1.5 mL/min. The linearity range for the proposed method was 0.25-50µg/mL. Photodiode array detector was set at a wavelength of 264nm to monitor the elution. The LOD and LOQ for thiocolchicoside were 0.05µg/mL and 0.125µg/mL respectively. The correlation coefficient ('r'^{2}) values were found to be >0.996 and % RSD values for intra-day and inter-day were found to be less than 10%. The method was validated for linearity, specificity, accuracy, precision, robustness, LOD and LOQ as per ICH guidelines and was suitable for estimation of thiocolchicoside in *ex-vivo* skin diffusate samples and transdermal patches.

Key-words: High Performance Liquid Chromatography, Thiocolchicoside, *Ex-vivo,* Transdermal Patches

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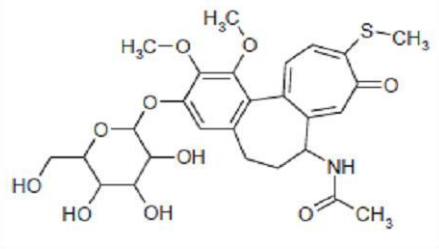
INTRODUCTION

Thiocolchicoside(THC) chemically,N-[(7S)-3-(beta-D glucopyranosyloxy)-1,2-dimethoxy-10-(methyl sulfanyl) 9- oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7yl] acetamide is a semi-synthetic derivative of the naturally occurring compound colchicoside.



Structure of Thiocolchicoside

It is a competitive GABAA receptor antagonist, acting as potent muscle relaxant with analgesic and anti-inflammatory properties. THC is available in tablet (4mg, 8mg&16mg), capsule (4mg & 8mg) and is administered once, twice or thrice daily depending on the maximum strength of the tablet to treat muscle spasm and for orthopedic and rheumatological disorders. When administered orally it results in low oral bioavailability of 25% due to first pass hepatic metabolism.[1, 2]

In order to improve the bioavailability an alternate route of administration in the form of transdermal or buccal are preferred. [3]

Literature survey reveals the estimation of thiocolchicoside by spectrophotometry, [4-12] HPLC, [13-20] HPTLC, [23-25] and by mass spectroscopic[26]methods individually or in combination of other drugs.The reported methods are applicable for estimation of thiocolchicoside individually or in combination of other drugs from pharmaceutical dosage forms. Till date no report has been published based on the HPLC method for the estimation of THC in ex-vivo samples from various transdermal therapeutic systems.

The aim of the present work is to develop a HPLC method for the estimation of THC in skin diffusate samples and estimation of THC content in transdermal patches. The advantage of the present method includes simple, sensitive, accurate, use of inexpensive chemicals and shot run time.

EXPERIMENTAL

Materials and Reagents

Thiocolchicoside was a gift sample from Sun Pharmaceutical Industries Ltd, India. Methanol (HPLC grade) was purchased from Merck, Mumbai, India. All other chemicals used were of analytical grade.

Chromatographic conditions

The analytical column used was Reverse phase C18 column (Inertsil ODS 3 V C-18, 250 × 4.6 mm, 5µ) at ambient temperature. The mobile phase used was Buffer: Methanol (45:55) (5.76 g Ammonium dihydrogen phosphate in 2000mL of milli-Q water, adjust pH 7.3 with tri ethyl amine) 10. µL of samples was injected to the HPLC system.

Preparation of Calibration standards

Primary stock solution of 1mg/mL of Thiocolchicoside was prepared in methanol. Quality control samples and standard solutions were prepared by the serial dilution of the prepared primary stock solution using pH 7.4 phosphate buffer saline (PBS) The calibration standards, 0.25, 0.5, 1, 2, 5,10,25,50, µg/mL and QC samples at three different levels (0.3, 15 & 30 µg/mL for THC) were prepared from corresponding stock solutions.

Preparation of Sample

The ex vivo permeated samples were filtered through 0.45 µm membrane filter, suitably diluted with pH 7.4PBS and was injected into HPLC. For the estimation of drug content in THC transdermal patches, the patch was cut into small pieces and was diluted with pH 7.4 PBS. The solution was adjusted to volume, filtered through 0.45 µm membrane filter and was injected into HPLC.

Method validation

Validation of an analytical method is the procedure by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the proposed analytical application of

International Conference on Harmonization (ICH) guidelines.[26] Validation is requisite for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation program required depends entirely on the particular method and its proposed applications. Typical analytical parameters used in assay validation include: linearity, range, precision, accuracy, limit of detection, limit of quantification, robustness, selectivity and specificity.

Linearity and range

Calibration curves were determined for eight different calibration standards, with a proposed range of 0.25-50µg/mL. Each calibration was injected three times. The calibration curve was performed in triplicate. Calibration curves were calculated applying the least-squares method to concentration versus peak area. Each plot as a linear relationship between concentration and peak area was observed in the range of study.

Precision

Precision, expressed in terms of %RSD, was determined in terms of intra-day and inter-day precision of the method was assessed in terms of repeatability by analyzing the drug at three different concentrations, determining each concentration thrice. The % coefficient variation (CV) values were expressed for intra-day precision and on 3 days for inter-day precision.

Accuracy

Accuracy was determined by performing recovery studies by the standard addition method by spiking different concentrations of pure drug in the solution containing THC within the analytical concentration range of the proposed method at three different set at level of 50%, 100% and 150%. QC samples at three levels were tested by spiking (0.3, 15 & 30 µg/mL).The amount of THC was calculated at each level and %recoveries were computed. The results were within the specified limits of ICH guidelines.

Specificity

Selectivity and Specificity of the method were determined by injecting pH 7.4PBS into the chromatograph and blank chromatogram was recorded. The blank chromatogram showed no peaks at the retention time of THC. The extraction recovery of THC was calculated by comparing the peak area ratio measured for the standard solution with that obtained for drug in skin permeated samples and Transdermal patches. Results from this testing studies indicated the method was enabled highly selective for analysis of the THC. Because the specificity or selectivity of a developed method is often difficult to ensure, several simple and easy techniques are used in method validation experiments to increases confidence in selectivity (peak purity). Results from these procedures confirmed the method was selective and specific.

Limits of detection (LOD) and limit of quantification (LOQ)

Sensitivity of the developed method was estimated by determining the LOD and LOQ of THC based on standard deviation of response and slope of calibration curves. The linearity study was carried out in triplicate.

$LOD = (3.3\ \sigma)/S$

$LOQ = (10\ \sigma)/S$

Where S is slope and σ is standard deviation.

Robustness

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for the routine analysis. Robustness was carried out by varying three parameters (deliberate change) from the optimized chromatographic conditions. To determine the Robustness of the developed method slight changes were made in the chromatographic conditions and results were examined. The flow rate, pH and composition of mobile phase were altered by 1.5 ± 0.1 mL/min, ± 0.2 and ± 2mL respectively. No significant change was observed and with all the conditions of robustness indicates that the developed method was robust and not affected by deliberate changes in the method parameters.

System suitability test

The system suitability was evaluated by calculating the % RSD values of peak area, retention time, asymmetry and theoretical plates of five replicate injections(5 µg/ mL) of THC. The column efficiency and peak asymmetry were calculated from standard solutions. The experimental results showed that the values were within the acceptable range indicating that the system was suitable for the intended analysis.

Application to *ex- vivo* skin permeated samples

The ex vivo permeation study of THC transdermal patches across the albino rat abdominal skin was conducted with the permission from the institutional ethical committee, Vaagdevi College of Pharmaceutical Sciences, Warangal, India. *Ex-vivo* permeation study was conducted using vertical Franz diffusion cells with a diffusional surface area of 3.54 cm^2. The rat abdominal skin was mounted between the donor and receptor compartment. The transdermal patch was placed on the skin surface. The receptor compartment was filled with pH 7.4 PBS as dissolution media. The entire assembly was placed on the magnetic stirrer and the contents of the receptor medium was mixed with stirring bars at a speed of 400 rpm and temperature was maintained at 37 ±2 °C. 1.5 mL of sample aliquots was collected at pre-determined time points and was injected into the HPLC system after filtering through 0.25 µm membrane filter and suitably diluting it. The permeation parameters and cumulative amount of the drug permeated across the skin was calculated.

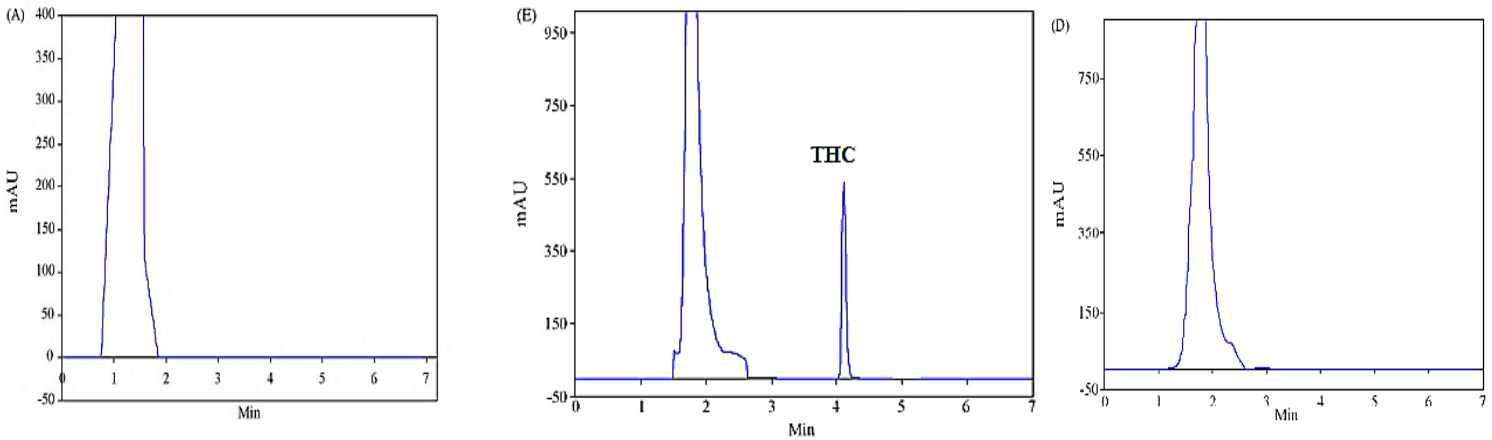
RESULTS AND DISCUSSION

Method development and optimization

The aim of present work was to develop and validate a method for estimation of THC by HPLC and the developed method was also applied for the estimation of THC in skin permeated samples and in Transdermal patches. Method development focuses on identifying and optimizing the mobile phase composition. Optimization of mobile phase composition was done by taking different proportions of aqueous and organic phases which gave acceptable asymmetry and theoretical plates with appropriate run time. From the different mobile phases tried mobile phase consisting of Buffer (Ammonium dihydrogen phosphate aqueous solution): Methanol (45:55, v/v) was found to be satisfactory. The pH of the mobile phase was also optimized as pH 7.3 using try ethyl amine, where the drug gave symmetric and sharp peak for THC at 1.5 mL/min as flow rate with good theoretical plates and acceptable tailing factor. For quantitative analytical purpose wavelength was set at 264 nm, which provided better reproducibility with minimum interference. Under the chosen experimental conditions, the liquid chromatogram of THC showed a single peak of the drug at retention time (RT) 4.12 min with asymmetry of 0.90 (**Figure 2).**

Chromatographic Method:

The chromatographic conditions and sample preparation for the proposed method were optimized. **Fig.2**shows typical HPLC chromatograms of (A) pH7.4 PBS (B) PBS spiked with 5µg/mL of THC (C) sample collected from ex vivo study at 4hr after application of drug (THC) loaded (4mg) Transdermal patch (D) THC free Transdermal patch (E) THC loaded Transdermal patch. The retention time of THC is 4.12 minutes with total run time of less than 7 minutes. System suitability parameters for the method are as follows: theoretical plates for THC was 2450 and tailing factor was less than 1.2



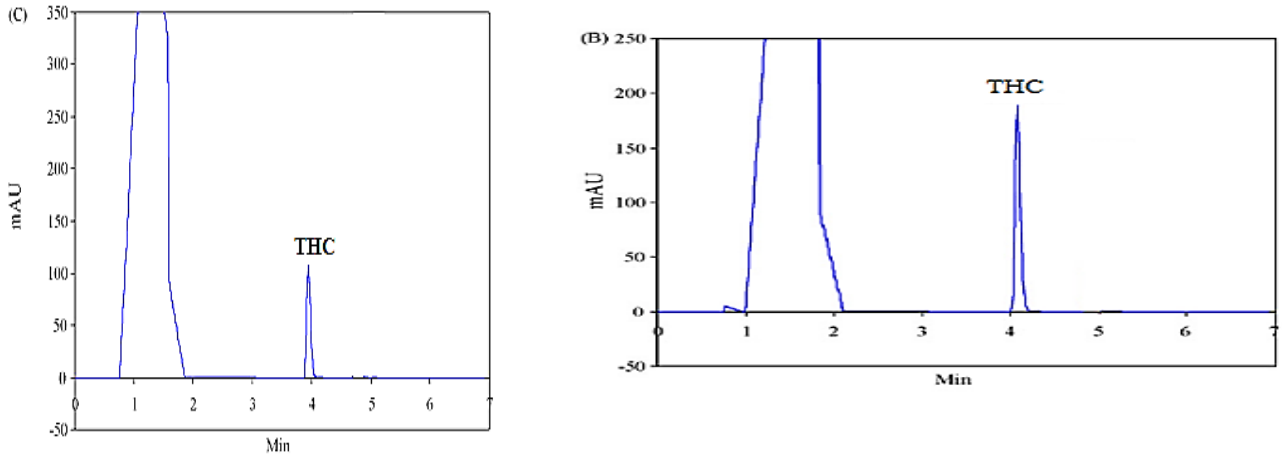


Fig-2 :HPLC chromatograms of (A) pH7.4 PBS (B) PBS spiked with 5µg/mL of THC (C) sample collected from ex vivo study at 4hr after application of drug (THC) loaded (4mg) Transdermal patch (D) THC free Transdermal patch (E) THC loaded Transdermal patch.

Method Validation:

Specificity:

The specificity of the HPLC method is illustrated in **Fig.2** where complete separation of THC was seen in presence of ex vivo sample or components of transdermal patches. The peak of THC was clear and no endogenous peaks observed at retention time of THC. The components of ex vivo samples or Transdermal patches did not interfere with THC thereby confirming the specificity of the analytical method.

Table 1: Linearity parameters for the estimation of THC

Parameter	THC
Linearity range (µg mL^{-1})	0.25-50
Slope	22511± 1.53
Intercept	3866 ± 40.41
Correlation coefficient	0.996±0.001

Values presented are mean ± SD of 3 calibration curve.

Linearity and range:

The calibration range was established by considering the practical range necessary for estimation of THC in skin permeated samples and Transdermal patches to give accurate and precise results with good linearity. The calibration curve was liner over the concentration range of 0.25 -30µg/mL. Regression analysis was carried out on calibration curve and results are summarized in **Table 1**indicating a good correlation with r > 0.996.

Precision

Precision was calculated as intra- and inter-day coefficient of variation (CV) as shown in **Table 2**.The percentage CV were found to be less than 3 and 4 for intra-day and inter- day precision respectively indicating that the method is reliable. The inter day precision was demonstrated on different days at three QC standards that cover the assay method range.

Accuracy

Accuracy of the method was studied by spiking samples of pH 7.4 PBS with reference standards. The mean recovery values for THC were shown in Table 2, indicating that the developed method was accurate for the determination of THC in skin diffused samples and Transdermal patches. The recovery was 100.1± 0.5 for all samples with % CV less than 5%

Table 2: Intra- day & inter- day precision and accuracy data for assay of THC n=5)

Added conc. (µg mL^{-1})	Calculated conc. (µg mL^{-1})		%CV		Accuracy	Range
	Intra -day	Inter- day	Intra-day	Inter -day		
0.3	0.3±0.006	0.29±0.01	2.65	2.09	99.2±1.2	99.3-101.2
15	15.7±0.06	15.4±0.43	1.27	3.85	102.5±1.4	100.9-103.8
30	30.3±0.34	29.4±0.28	1.16	1.52	100.3±0.8	99.1-101.3

Limits of detection (LOD) and limit of quantification (LOQ)

The parameters LOD and LOQ were determined on the basis of signal to noise ratio, LOD and LOQ were calculated by the method which was based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approximating the LOD and LOQ were found to be 0.05 & 0.125 µg/mL respectively.

Robustness

The robustness of the method was evaluated by assaying the same sample under different analytical conditions deliberately changed from the original analytical condition. The results obtained by the varying the conditions of flow rate, pH and composition of mobile phase had no impact on chromatographic performance and were in accordance with results of original conditions. The tailing factor of less than 1.2forTHC indicates that change in flow rate; pH and mobile phase composition have no impact on the chromatographic performance. Consequently with all the conditions of robustness indicates that the developed method was robust and not affected by deliberate changes in the method parameters.

Table -3:Robustness data of developed HPLC method

Parameter	Variations	RT	Tailing factor	Plates
Flow rate mL/min (± 0.1)	1.4	4.03	0.8	1685
	1.5	4.11	0.96	2420
	1.5	3.84	0.83	2119
pH ((± 0.20)	7.1	3.84	0.78	2222
	7.3	4.12	0.98	2538
	7.5	3.82	1.04	1963
Mobile phase (± 2 mL Methanol) (Buffer: Methanol)	47: 53	3.84	0.7	1908
	45 : 55	4.15	0.94	2448
	43 : 57	3.89	0.86	2160

Ex-vivo skin permeation study and drug content estimation in transdermal patches.

The developed method was applied for estimation of THC in skin transfused samples and in Transdermal patches. The **Fig- 3** shows the cumulative amount of THC permeated from the patch across the albino rat abdominal skin. The drug content in the patch was found to be 3.95 mg, about 1071µg of THC was permeated in 24hr with the flux of 0.38.µg hr^{-1}cm^{-2}

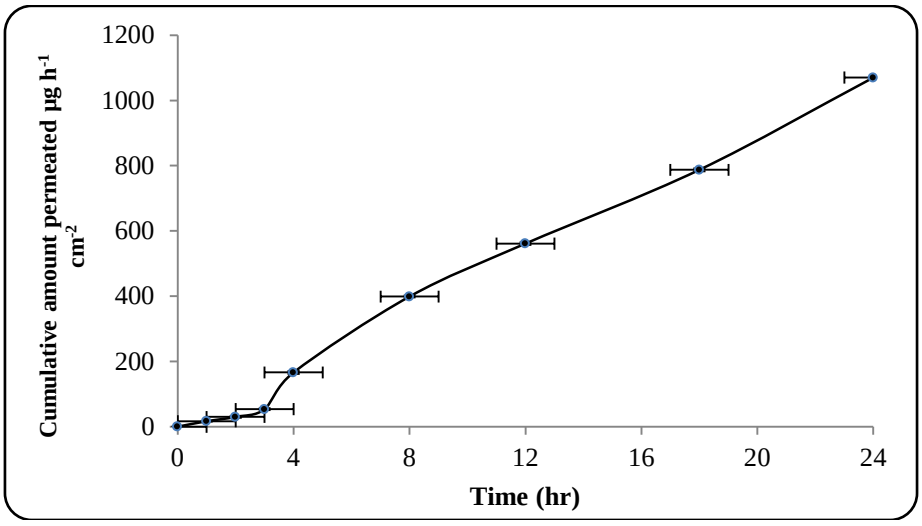


Fig- 3 : Ex-Vivo permeation profile of Thiocolchicoside, results represented are mean ±SD (n=3)

CONCLUSIONS:

A simple and sensitive HPLC method for the estimation of THC in ex-vivo samples was developed and validated. No interfering peaks were observed at elution time of THC. The developed HPLC method was specific, accurate reproducible and was suitable for the analysis of THC in skin diffusate samples from Transdermal patches across rat abdominal skin

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