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Quantification of Thiocolchicoside in Rabbit Serum by High Performance Liquid Chromatographic Method: Application to Pharmacokinetic Study

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Abstract

Objective: A sensitive and précised bioanalytical method for quantification of Thiocolchicoside (THC) in rabbit serum was developed using high performance liquid chromatographic technique. Aceclofenac (ACE) was used as Internal Standard (IS). **Methods:** Phenomenix Luna® C18 column (150 x 4.6mm.i.d.5 µ) was used for method development of THC.Ammonium di hydrogen phosphate buffer: Acetonitrile (60:40) was used as mobile phase where in pH was adjusted to 4. 5 with glacial acetic acid. **Findings:** Flow rate was set to 1.0 mL/min and elements were monitored at 264 nm by UV detector. The retention times detected were 3.16 and 4.25 minutes for THC & ACE respectively. Linearity of the calibration curve was in the range of 10- 4000 ng mL^{-1}. Inter day, intra-day coefficient of variation and percent error values of the developed assay method was with the acceptable limits of less than 15 %. The mean recovery was recorded to be more than 98.6 and 99.5 % for THC and ACE respectively. **Application:** Validated bio-analytical method was successfully applied for estimation of THC in Rabbit serum after application of Transdermal films to the rabbit skin.

Keywords: Pharmacokinetics, Rabbit Serum, Thiocolchicoside, Transdermal films

1. Introduction

Chemically Thiocolchicoside Figure 1 is N - [3 - (β - D - glucopyranosyloxy) - 1, 2 - dimethoxy - 10 (methylthio) - 9 - oxo - 5, 6, 7, 9 - tetrahydrobenzo [*a*] heptalen-7-yl] acetamide.[2] Thiocolchicoside (THC) is a semi-synthetic derivative of colchicines;it has an affinity for the inhibitory Glycine and gamma-aminobutyric acid (GABA)-A receptors.[2] It is used in the treatment of rheumatologic, traumatic and orthopedic disorders[3]. THC is used as muscle relaxant agent without any side effects, its safety and efficacy is demonstrated in many clinical trials.[1,2,4-8]

THC rapidly absorbed from the gastrointestinal tract, undergoes first pass metabolism with an oral bioavailability of about 25 % and a biological half-life of 5-6 h. Thiocolchicoside is broken down in the body to a metabolite called 3-demethylthiocolchicine that could damage cells therefore inducing toxicity in the embryo, neo-plastic changes and fertility reduction in male.[16] Local skin preparations are less toxic.[16] Thiocolchicoside is available in the market as capsule, tablet and injection under the brand names Bakflex, Colcoside, Mofree, Mobiwork, Lupiflex, Thiospas, Myoril and Periset respectively.

Analytical methods are reported for the estimation of THC in Human plasma and serum based on LC-MS and RP-HPLC.[9-11] In this study we have made an attempt to develop and validate a HPLC method where in drug can be estimated in nanograms when THC formulations are applied transdermally to rabbits.

THC is a skeletal muscle relaxant with mild sedative effect thus rabbits are used as animal model for conducting pharmacokinetic study. The present study aims at

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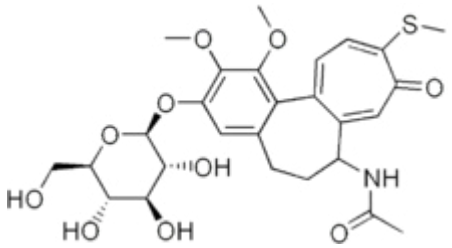
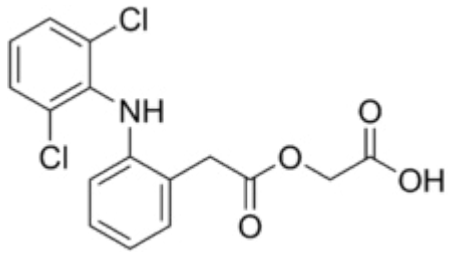
estimation of THC concentrations after administration of the optimized drug embedded matrix type transdermal film by developing a sensitive, reliable and robust bio-analytical method in rabbit serum and its applications to pharmacokinetic study. The developed method has many advantages of being sensitive, simple extraction procedure, use of inexpensive chemicals, less organic solvent consumption and short run time.



(a)



(b)

Figure 1. Structure of (a) Thiocolchicoside and (b) Aceclofenac.

2. Materials and Methods

Thiocolchicoside and Aceclofenac pure samples were gifted by Dr. Reddy's laboratories, Hyderabad, India. Glacial acetic acid, ammonium di hydrogen phosphate analytical grade and Acteonitrile HPLC grate for analysis were purchased from Merck, Mumbai, Maharashtra, India. Triple distilled water was used throughout the development and validation of HPLC method.

2.1 Chromatographic Conditions

The HPLC system consisted of a LC-2010 AT solvent module, SPD 10A UV- visible detector with LC 10 software.

Analysis was carried on Phenomenix Luna® C18 column (150 x 4.6 mm i.d. 5 µ).Ammonium di hydrogen phosphate buffer : Acetonitrile (60:40) used as mobile phase where in the pH was adjusted with glacial acetic acid to 4.5. The flow rate of 1.0 mL/min and 50 µL volume of injection was used to monitor the eluent at 264 nm. The detector sensitivity was set to 0.005 AUFS.

2.2 Preparation of Calibration Solutions

Primary stock solutions 1 mg mL^{-1} each were prepared by dissolving 10 mg of THC and internal standard ACE Figure 1 in 10 mL methanol.[17] These were further diluted to get working stock solution of 10 µgmL^{-1}. Stock solutions were refrigerated at 4°C until used. The concentrations of 10, 25, 50, 100, 250, 500, 1000, 2000 & 4000 ng mL^{-1} were prepared for construction of calibration curve. 100 µL of these concentrations were spiked to 0.5 mL of rabbit serum to get calibration solutions.

2.3 Preparation of Standard Quality Samples

Three different levels of standard quality samples were prepared by spiking 100 µL of 25, 1750 and 3250 ng mL^{-1} concentrations to 0.5 mL of rabbit serum.

2.4 Preparation of Plasma Samples

Fractional volume of 0.5 mL blank plasma spiked with THC is taken in to screw capped tubes and 100 µL of An Internal Standard (ACE) 1000 ng mL^{-1} was added and both were extracted with 5.0 mL of butyl acetate. The tubes were subjected for 15 min shaking at 50 rpm and then centrifuged for 10 min at 4000 rpm. The organic layer was separated and was transferred to a conical flask. The aqueous layer was re extracted with 5 mL butyl acetate. Both organic and aqueous layers were mixed and were evaporate in vacuum oven at 40 °C. The residues were reconstituted in 150 µL mobile phase. A volume of 50 µL was injected into HPLC.

2.5 Validation Parameters

Validation was carried out as per ICH & FDA guidelines.[12-15] Sample concentrations of 25, 1750 and 3250 ng mL^{1} were injected for estimation of precision and accuracy. Assay results were determined by percentage coefficient of variation and percent relative error. Samples were prepared according to the method descried in preparation of plasma samples, six sets of samples were prepared for 5 days to study intra and inter day precision and accuracy. LOD was determined from the signal to noise value of 10 :1 by comparing the results of the test samples with samples of known concentration of analytes with blank samples.

2.6 Recovery Studies

The standard quality samples of 25, 1750 and 3250 ng mL^{-1} were extracted from the above mentioned procedure of preparation of plasma samples and replicates of five were injected into the HPLC system. The extraction recovery at each concentration was determined by using the formula given below.

Recovery = (Peak area obtained after extraction/peak area obtained after direct injection) X 100

2.7 Stability Studies

Standard quality samples at three different levels 25, 1750 and 3250 ng mL^{-1} were subjected to stability studies to check the reliability of handling and storing of stock solutions and serum samples. Six replicates of each concentration were prepared, kept at room temperature followed by refreezing for 24 h at -20°C to study the freeze thaw stability. THC and ACE stock solution stability at a concentration of 500 ng mL^{-1} was carried out at room temperature and upon refrigeration for 24 h and 15 days respectively. Long term stability of spiking rabbit serum was checked by storing samples for 24 h at room temperature and refrigerating it for 20 days at -20 °C. Stability was ensured by comparing the final assay values of samples with initial assay value of drug.

2.8 Robustness

Robustness was check by purposely altering percent organic strength (40 ± 2 %), flow rate (1 ± 0.2 mL), and pH of the buffer (4.5 ± 0.2) by keeping the mobile phase ratio constant.

2.9 Application to Pharmacokinetic Study

With the permission of the Institutional the Animal Ethical committee, (IAEC/13/VIPS/2016) Vaagadevi Institute of Pharmaceutical Sciences, Warangal, India, the pharmacokinetic study was conducted in six healthy rabbits (body weight 2.5 ± 15 kg). Pharmacokinetic parameters maximum plasmaconcentration (C_{max}), time to reach maximum plasma concentration (t_{max}), area under the curve (AUC) and half-life ($t_{1/2}$) were calculated using computer program KINETICA 2000 (Version 3.0, Inner phase corporation, Philadelphia, USA) for each rabbit.

Matrix type transdermal film of THC was applied to the healthy rabbits, from marginal ear vein blood samples were collected for 72 h, processed with suitable extraction procedure and were then analyzed by HPLC.

3. Results and Discussion

3.1 Chromatography

The HPLC method appears to be the most feasible for accomplishing lower limit of quantification and detection. Since patients are exposed to a wide variety of drugs, it is necessary to develop methods able to quantify lower concentrations. Although there is a large demand for analytical methods that accurately determine various compounds in biological and non-biological matrices. Current day literature emphasises, methods for estimation for THC in non-biological samples with minimum detection limits of 100 ng mL^{-1}. In this study, the aim was to develop a method able to quantify the very low plasma concentrations 10 ng mL^{-1} presence in rabbit serum.

3.2 Calibration Curve

The linearity range was 10 ng mL^{-1} to 4000 ng mL^{-1}.The regression equation obtained from 9 points was y=46469x – 19225 with a correlation coefficient of 0.9974.

3.3 Specificity and System Suitability

The chromatographic conditions of recommended method were adjusted to outfit the below pharmacokinetic studies. Figure 2 reflects typical chromatograms of blank rabbit (drug free) serum; Figure 2 shows the chromatogram of blank serum spiked with THC to a concentration of 10 ng mL^{-1} and internal standard, Figure 2 shows chromatogram of rabbit serum sample at 24 h after application of transdermal film containing 4 mg of THC. The retention times were found to be 4.25 and 3.16 min for THC and internal standard respectively with a run time of less than 10 min. The chromatogram revealed that they are well separated from each other under the applied chromatographic conditions. The entire analytical process of THC and ACE were resolved with a good symmetry. The absence of interfering peaks in the individual blank serum and the spiking samples at the time of retention of drug and internal standard accomplishes specificity of developed bio-analytical method.

The system suitability parameters like theoretical plates and tailing factor observed to be within the limits and the plate count for THC and ACE were recorded as 6234 and 1151 respectively with tailing factor less than 1.2 and resolution of 7.0.

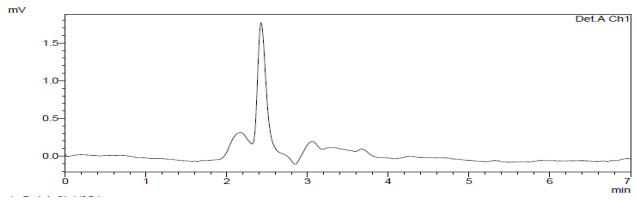


Figure 2 (a). Chromatogram of blank rabbit serum.

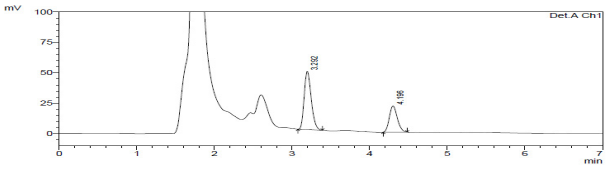


Figure 2 (b). Chromatogram of blank serum spiked with THC to a concentration of 15 ng mL^{-1} and internal standard.

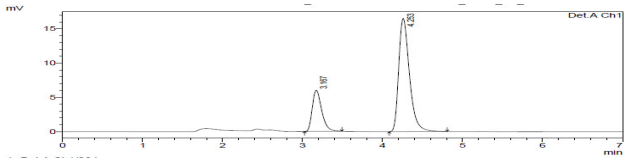


Figure 2 (c). Chromatogram of rabbit serum sample at 24 h after application of transdermal film containing 4 mg of THC.

3.4 Precession and Accuracy

Standard quality samples at three different levels were prepared and were subjected to intra-day and inter day precision and accuracy for five days. Satisfactory results were achieved with % CV less than 15 %, according to the CDER guidance document for bio-analytical method validation and accuracy with % relative error within ± 1 % was recorded and tabulated in Table 1.[14]

3.5 Limit of Quantification and Limit of Detection

Spiking Calibration solutions were prepared in replicates of six and concentrations were determined to establish LOQ. The recorded value was 5 ng mL^{-1} for THC in rabbit serum. %CV was less than 20% with an accuracy of 85 to 110 %.The LOD was determined to be 2.5 ng mL^{-1} based on s /n ratio of 4 :1.

3.6 Percentage Recovery

Peak areas of standard quality samples were compared with calibration solutions to determine recovery. The recovery of THC was 95.5, 96.8 and 97.9 % respectively for standard quality samples at concentrations of 15, 2750 and 3250 ng mL^{-1}. Mean recovery of internal standard was 96.8 ± 1.43 %. Results were represented in Table 2 and 3.

3.7 Stability

The stability study results are represented in Table 4. About 97 % of THC and ACE were remained in calibration solution revealing the stability of analytes for at least

Table 1. Intra day and inter day precision and accuracy studies for assay of THC in rabbit serum (n=6)

Parameters	Added concentration ng mL^{-1}	Calculated concentration ng mL^{-1}	% CV	% Error
Intra day	15	14	11.1	-0.02
	1750	1746	1.7	0.01
	3250	3253	1.1	0.02
Inter day	15	15	9.2	0.70
	1750	1748	2.1	-0.07
	3250	3246	0.8	-0.12

Table 2. THC Recovery studies

Thiocolchocoside	Standard drug Concentration ng mL^{-1}	Amount of drug recovered ng mL^{-1} Mean ± SD	% Mean ± SD (n=6)	Range (Min-Max)	% CV
Recovery studies	15	14±0.3	95.5±3.8	92.4-99.3	5.4
	1750	1738±12.5	96.8±1.1	95.6-98.8	1.0
	3250	3230±20.4	97.9±0.9	96.3-100.2	0.8

Table 3. THC Accuracy studies

Thiocolchicoside	Accuracy Studies		
Standard drug Concentration ng mL^{-1}	15	1750	3250
% Accuracy Mean ± SD (n=6)	96.7 ± 2.3	96.4 ± 1.3	96.3 ± 1.8
Range (Min-Max)	88.7 - 101.1	96.2 - 98.6	95.4 - 98.8
% CV	6.8	1.8	1.3

Table 4. Stability of THC in Rabbit serum

Stability	Spiked concentration (ngmL^{-1})	Calculated sample concentration (ng mL^{-1})		Calculated stability sample concentration (ng mL^{-1})		Average (%)
		Mean ± SD	% CV	Mean ± SD	% CV	
Work table stability[a]	15	15 ± 0.2	1.8	15± 0.2	2.0	97.0
	1750	1775± 10.2	1.2	1765 ± 15.3	1.7	96.8
	3250	3263 ± 12.5	0.5	3255 ± 18.5	1.1	97.5
Freeze and thaw[b]	15	15 ± 0.2	2.0	15± 0.2	2.8	98.1
	1750	1747± 12.3	0.9	1740 ± 16.1	2.1	97.6
	3250	3253 ± 13.8	0.3	3246 ± 22.6	1.6	96.5
Extended term[c]	15	15 ± 0.3	2.5	15± 0.4	8.8	98.0
	1750	1760± 16.9	2.4	1743 ± 14.4	1.3	97.5
	3250	3252 ± 22.2	0.6	3246 ± 8.5	0.8	96.2

[a]After 10 h at room temperature;
[b]After freez thaw cycles;
[c]After 20 days at-20 °C .Values are mean ± SD (n=6)

Table 5. Method robustness data of THC in Rabbit plasma

Parameter	Modification	RT (Min)		Tailing factor		Plates		Resolution
		THC	ACF	THC	ACF	THC	ACF	
Mobile phase Ratio (v/v Buffer : ACN)	68: 32	3.52	5.27	1.14	1.12	6573	14642	6.53
	60: 40	3.16	4.25	0.10	0.85	6126	12845	7.50
	52: 48	2.94	4.10	1.10	0.98	5652	11891	7.89
Flow rate (mL/ min)	0.8	5.26	6.27	1.21	1.13	9276	18845	6.63
	1.0	3.20	4.26	0.11	1.00	6199	12684	7.49
	1.2	2.14	2.25	0.92	0.88	4573	9383	7.90
pH	4.3	3.09	4.10	1.08	0.98	5785	11989	7.32
	4.5	3.18	4.24	1.00	0.93	6055	12534	7.52
	4.7	3.21	4.35	1.14	1.05	6114	12709	9.82

20 days when stored at room temperature for 10 h at 4 °C. THC was stable for 8 h in rabbit serum can be stated from the bench top stability study. The thawing and freezing of serum samples spiked with THC and long term stability study at three levels, showed more than 96 %.

3.8 Robustness

The method was robust and unaffected by variations made in mobile phase composition (organic phase), buffer concentration, flow rate and pH of buffer had no impact on chromatographic performance. The resolution

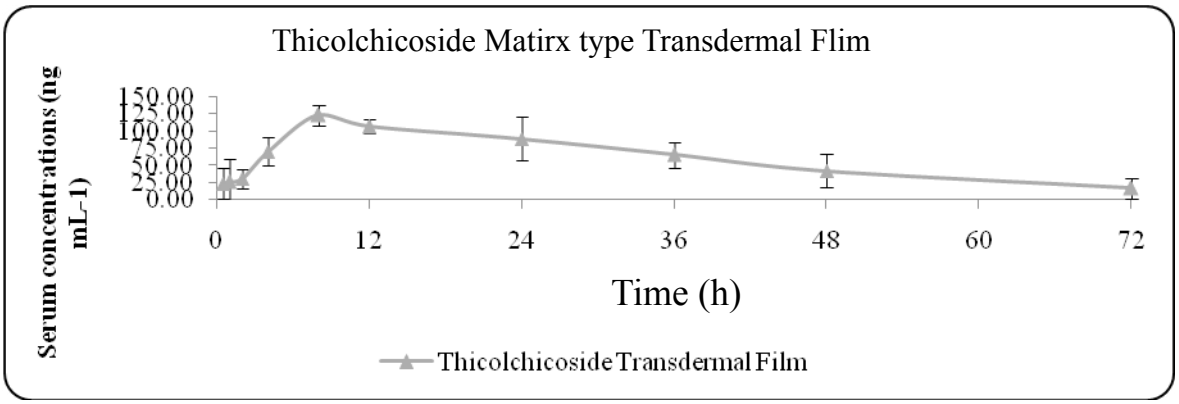


Figure 3. Mean serum concentration-time profile of THC in rabbits (n=6) after application of THC matrix type transdermal film. Values represented are mean ± SD.

ranged between 6.53 to 9.82.The tailing factor was less than 3 for both THC and ACE and both analytes were well separated under all the changes that were made. Results presented in Table 5. The studied parameters were within the limits.[14]

3.9 Application to Pharmacokinetic Study

The developed method can be used for quantification of THC in rabbit serum obtained after the application of matrix type Transdermal film containing 4 mg drug. Maximum plasma concentration for THC reached at 8.12 ± 2.5 h and was calculated as 122.85 ± 25.3 ng mL with AUCof 896.27 ± 376.85 ng hr mL^{-1}. Pharmacokinetic parameters are represented in Table 6 and Figure 3 shows the plasma concentration time curve of THC.

Table 6. Pharmacokinetic parameters of thiocolchicoside after application of matrix type transdermal film to rabbit (n=6)

Formulation	Matrix type transdermal film
$C_{Max\ (ng\ mL^{-1})}$	122.85 ± 25.3
$T_{Max\ (h)}$	8.12 ± 2.5
$AUC_{0\text{-}t}$ (ng h mL^{-1})	896.27 ± 376.85
$AUC_{0\text{-}total}$ (ng h mL^{-1})	982.63 ± 352.42

4. Conclusion

A simple, precise and sensitive HPLC method was developed for quantification of THC in rabbit serum. The method was inexpensive, reliable and showed no interfering peaks at the retention time of THC and ACE. The established method was suitable for estimating the pharmacokinetic parameters of THC in rabbits.

5. Acknowledgements

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