

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF THIOCOLCHICOSIDE AND LORNOXICAM IN HUMAN PLASMA AND IN PHARMACEUTICAL DOSAGE FORM BY RP-HPLC

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ABSTRACT

Combination therapy of Lornoxicam (LOR) and Thiocolchicoside (THC) is used in pain management. To obtain optimal drug concentrations (minimum effective concentrations) for pain suppression and avoidance of drug toxicity, monitoring of drug levels has been considered essential to determine drug concentrations in plasma. A new reverse phase high-performance liquid chromatography (RP-HPLC) assay was developed for the simultaneous determination of Lornoxicam and Thiocolchicoside in human plasma as well as in pharmaceutical dosage form. The method involves a simple protein precipitation procedure that uses no solid-phase or liquid-liquid extraction. The compounds were separated by using a mobile phase of Phosphate buffer (pH 6.8) and Acetonitrile (70:30 v/v) in isocratic flow with flow rate 1 ml/min on a Phenomenex Luna S-C18 column (5 µm, 250mm X 4.60mm i.d.) with PDA detection at 295 nm. The retention time for Thiocolchicoside is 5.94 ± 0.2 min, for Lornoxicam 14.53 ± 0.2 min. Validation of the method was performed in terms of linearity, accuracy, and precision, limit of detection and limit of quantification.

The method was validated over the range of 100-500 ng/ml for Thiocolchicoside ($r^2 = 0.9983$) and 200 -1000 ng/ml for Lornoxicam ($r^2 = 0.9972$) for both plasma and tablet dosage form. The limit of detection was 33.27 ng/mL for Thiocolchicoside and 66.2 ng/ml for Lornoxicam in human plasma, and the lower limit of quantification (LLOQ) of Thiocolchicoside and Lornoxicam in plasma was 100.82 and 200.48 ng/ml respectively. Extraction recoveries of the analytes in plasma were ranging from 83.43-91.57 % at three different levels of concentration (LQC, MQC, and HQC). So the assay is suitable for use in pharmacokinetic studies and routine plasma monitoring of this combination drug therapy in patients of musculoskeletal disorders.

Keywords: RP-HPLC, LOR, THC, LQC, MQC and HQC.

INTRODUCTION

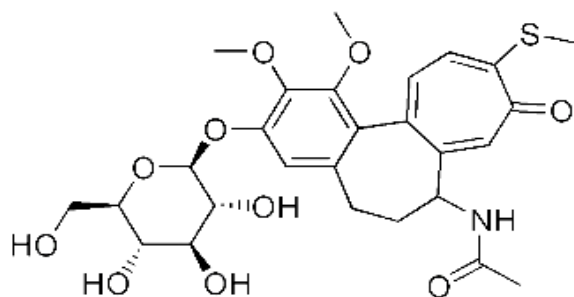
According to World Intellectual Property Organization (WIPO), in the treatment of musculoskeletal disorders as well as in the treatment of pain and inflammation in mammalian organism, a pharmaceutical composition should comprise effective amount of one muscle relaxant and at least one nonsteroidal anti-inflammatory drug. A combination therapy involving Thiocolchicoside (THC) and Lornoxicam is introduced in market for the treatment of musculoskeletal disorders.

Thiocolchicoside (THC) chemically, N-[3-(β-D-glucopyranosyloxy)-5,6,7,9-tetrahydro-1,2-dimethoxy-10-(methylthio)-9-oxobenzo [a] heptalen-7yl] acetamide, **Fig.1.A**. It has selective affinity for γ-aminobutyric acid (GABA) receptors and acts on the muscular contracture by activating the GABA inhibitory pathways thereby acting as a potent muscle relaxant¹. Thiocolchicoside is not official in any pharmacopoeia. On detailed literature survey, it was found that Thiocolchicoside can be estimated by spectrophotometry²⁻³, HPLC⁴⁻⁶ and HPTLC⁷ methods individually or in combination with other drugs⁸⁻¹¹.

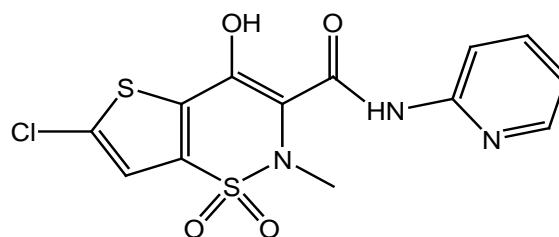
Lornoxicam is (6-chloro-4-hydroxy-2-methyl-N-2-pyridinyl-2H-thieno[2,3-e]1,2-thiazine-3-carboxamide 1,1-dioxide, **Fig.1.B**) a non-steroidal anti-inflammatory drug with analgesic and antipyretic properties that belongs to the class of oxicams. It acts by nonselective inhibition of cyclooxygenase-1 and 2. It is prescribed for osteoarthritis, rheumatoid arthritis, acute lumbar-sciatica conditions and postoperative pain management¹².

In the literatures, a voltammetric¹³, polarographic¹⁴, UV spectrophotometric¹⁵, LC/MS/MS¹⁶⁻¹⁷, HPTLC¹⁸, and high performance liquid chromatographic (HPLC)¹⁹⁻²² methods were reported for the analysis of Lornoxicam.

However, no method has been reported for the simultaneous determination of Thiocolchicoside (THC) and Lornoxicam mixture in human plasma. So we have developed and validated an isocratic HPLC assay with PDA detection for the simultaneous determination of THC and LOR in human plasma. The method is potentially suitable for drug monitoring, determination of pharmacokinetic profiles and for the determination of active ingredients in tablet dosage forms.



A. Thiocolchicoside



B. Lornoxicam

Fig. 1: Structure of Thiocolchicoside (A) and Lornoxicam (B)

MATERIALS AND METHOD

Chemicals and reagents

Pure samples Thiocolchicoside (THC, 99.56%) and Lornoxicam (99.45%) were obtained as a kind gift samples from Alembic Ltd., Vadodara, India. HPLC grade Acetonitrile and ortho phosphoric acid were procured from Merck Ltd., India. Highly pure water was prepared by using Millipore system. Human plasma is obtained from Institute of Medical Science (IMS), Varanasi, India.

Instrumentation

A High performance liquid chromatography system LC- 10AT_{VP} Shimadzu with photo diode array detector (SPD-M10 AVP-Shimadzu) was used for analysis. The data was recorded by using the software CLASS-LC10/M10A. The column used for separation was octadecyl silane (C₁₈) with length 250mm, internal diameter 4.6mm and particle size 5 μ (Phenomenex Luna S).

Chromatographic conditions

The chromatographic analysis was performed by using a mobile phase of Phosphate buffer (pH 6.8) and Acetonitrile (70:30 v/v) in isocratic flow with flow rate 1 ml/min on a Phenomenex Luna S -C18 column (5 μ m, 250mm X 4.60mm i.d.) with PDA detection at 295 nm.

A sample volume of 20 μ l was injected in triplicate onto the HPLC column. The chromatograms were recorded.

Preparation of standard stock solution

Standard stock solutions of 500 & 1000 μ g/ml were prepared for THC & LOR respectively by dissolving appropriate drug in Acetonitrile (stock A & B). From each stock solutions 1.0 ml aliquot was taken and diluted up to 100 ml to obtain the concentration of 5 μ g/ml and 10 μ g/ml for THC & LOR respectively. (stock C).

Preparation of blank plasma

To 1ml of plasma 4 ml of Acetonitrile was added, mixed thoroughly and left to stand for 5 min at room temperature. After 5 min the solution was centrifuged at 10000 rpm for 12 min at 10°C. The clear supernatant liquid was removed, filtered through 0.22 μ syringe filter and injected directly into HPLC system.

Preparation of standard plasma stock solution

To prepare calibration standards and quality control samples, appropriate quantities of the various diluted standard solutions added to blank plasma to obtain drugs in the concentrations range of 100-500 & 200-1000 ng/ml for THC and Lornoxicam respectively. (Table No. 1)

Table 1: Table shows Calibration spiking chart

S. No.	Spiked plasma Conc.(stock C)	Volume of stock C(ml)	Total Vol. (ml)	Plasma Conc. (ng/ml)	
				THC	LOR
1.	5:10 μ g/ml	0.2	10	100	200
2.	5:10 μ g/ml	0.4	10	200	400
3.	5:10 μ g/ml	0.6	10	300	600
4.	5:10 μ g/ml	0.8	10	400	800
5.	5:10 μ g/ml	1.0	10	500	1000

System suitability parameters

After setting the chromatographic conditions, mobile phase was allowed to saturate the column with flow rate 1 ml/min. After

complete saturation of column, six replicates of plasma standard of THC (500 ng/ml) and LOR (1000 ng/ml) were injected. Peak report and column performance were given in Table no.2.

Table 2: Table shows System suitability parameters of THC and LOR

System suitability parameter of THC (n=6 replicates, 500 ng/ml)				
	Retention Time	AUC	Theoretical plate	Tailing factor
Mean	5.9	136232	5714	1.19
S.D.	0.057	1002.33	84.25	0.07
R.S.D.	0.96	0.7358	1.47	0.058
System suitability parameter of LOR (n=6 replicates, 1000 ng/ml)				
Mean	14.52	237303	4526.3	1.17
S.D.	0.042	2710.4	139.52	0.037
R.S.D.	0.287	1.142	3.08	3.12

Bioanalytical Method Validation: The developed method was intensively validated following bioanalytical guidelines²³⁻²⁴. Bioanalytical method validation required the determination of LOD, LOQ, selectivity, linearity range, accuracy, precision, recovery and stability respectively.

Selectivity: To evaluate the Selectivity of the method, chromatogram of drug-free plasma sample was obtained, and retention times of the endogenous compound in the plasma were compared with those of THC and LOR.

Limit of Detection: For determining the limit of detection of the proposed method, Plasma samples were spiked with decreasing concentrations of the analytes and analyzed. The lowest concentration solution that shows characteristics similar to the reference chromatogram was considered to be the lowest detectable dilution.

$$\text{LOD} = 3.3 \sigma/S$$

Where σ = the SD of the response and S = the slope of the calibration curve.

Lower limit of Quantification

The limit of quantification (LOQ) was calculated to be the lowest analyte concentration in plasma that could be measured with relative standard deviation (RSD) of <20% and an accuracy between 80 and 120%.

$$\text{LOQ} = 10 \sigma/S$$

Where σ = the SD of the response and S = the slope of the calibration curve

Linearity of plasma samples

To establish the linearity of analytical method, a series of dilution ranging from 100-500 ng/ml for THC and 200-1000 ng/ml for LOR were prepared and calibration graph was plotted between the mean peak area vs. respective concentration and regression equation was derived.

Accuracy: - Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. The method was found to

be accurate if % nominal concentration is $100 \pm 15\%$ and 75 % calibration points are within the limit.

Precision: - The precision of the method based on within day i.e. repeatability was determined by replicate analysis of sets each of high, middle and low quality control samples. The reproducibility (day-to-day variation and analyst to analyst) of the method was validated by using similar six sets of high, middle and low quality control samples on different days and by different analyst.

Recovery: - In order to calculate recovery of extraction procedure, three replicates of LQC, MQC and HQC were prepared and compared with same concentration of un- extracted samples of THC and LOR.

Percent recovery: Mean peak response (extracted) X 100

Mean peak response (unextracted)

Stability

➤ **Bench top stability:-** Three replicates each of LQC and HQC were kept on bench at room temperature for approximately 24 hrs. Stability samples were processed and analyzed.

➤ **Freeze and thaw stability:-** The stability of plasma samples after three freeze-thaw cycles was also examined. The two concentrations used in the bench top stability study were assayed in triplicate over a period of three days. After each analysis, the samples were re-frozen until the next day. After three freeze-thaw cycles, the results were compared to the initial fresh unfrozen samples.

The method will be acceptable if % Change is $\pm 10\%$ and % CV $\leq 15\%$.

Method Validation for pharmaceutical dosage form

The proposed HPLC method was validated as per ICH guidelines for estimation of drugs in pharmaceutical dosage form²⁵.

Linearity: Linearity was studied by preparing standard solutions at different concentration levels as mentioned in table no.1 by using diluent Acetonitrile and linear regression equation was derived after plotting the calibration curve.

Accuracy: Recovery studies were performed to validate the accuracy of developed method. To the preanalysed sample solution, a definite concentration of standard drug was added and then its recovery was analyzed.

Precision

Repeatability: Dilutions of different concentrations were prepared and triplicates of each dilution were analyzed in same day for repeatability and the results were subjected to statistical analysis.

Intermediate Precision: In these, triplicates of each dilution were analyzed in different days and by different analysts.

Robustness: As per ICH norms, small variations like pH alteration or flow rate alteration or change in detection wavelength were made to check the method's capacity to remain unaffected.

RESULT AND DISCUSSION

Under the chromatographic conditions described, peaks of THC and LOR were well resolved. Endogenous plasma components and excipients of tablet dosage form did not give any interfering Peaks. The retention times for THC and LOR were 5.94 ± 0.2 min and 14.53 ± 0.2 min, respectively. Peak of plasma components was appear at 2.79 ± 0.2 min. Representative chromatograms are shown in Fig. 3,4,5,6.

Wavelength selection: THC exhibited two peaks at 260 and 290 nm (shoulder), while LOR also exhibited two peaks at 252 and 280 nm (Figure 2). Based on the spectral characteristics and overlay spectra, 295 nm was selected as the wavelength for detection because both the drugs showed considerable absorption at this wavelength.

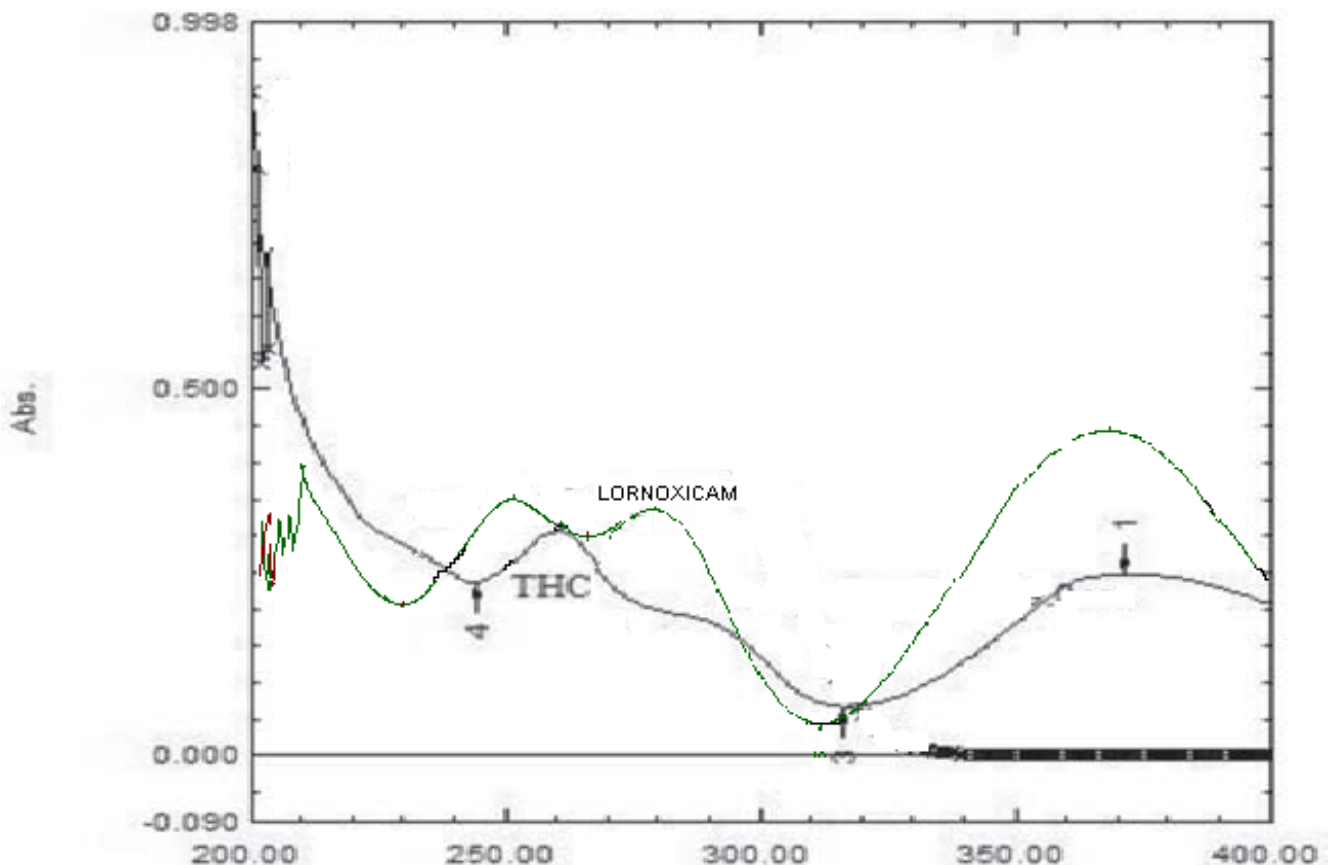


Fig. 2: It shows Overlay spectra of Thiocolchicoside and Lornoxicam

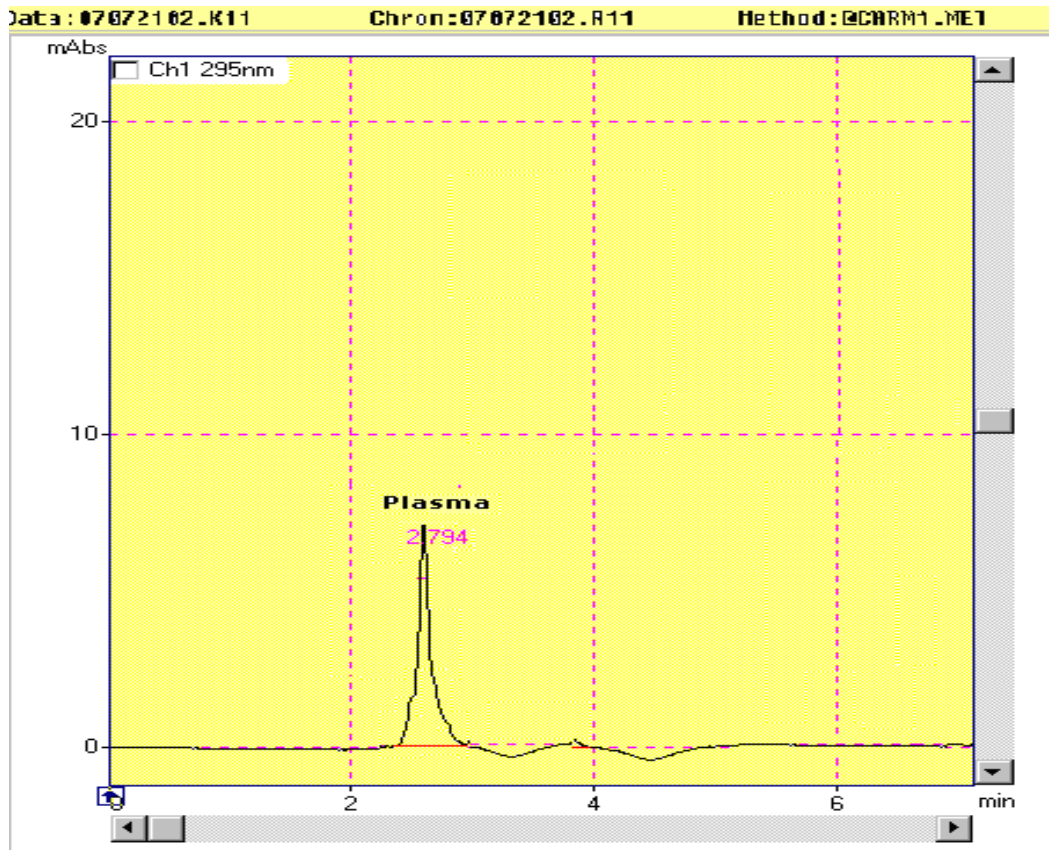


Fig. 3: It shows chromatogram of extracted blank plasma with retention time 2.794 min.

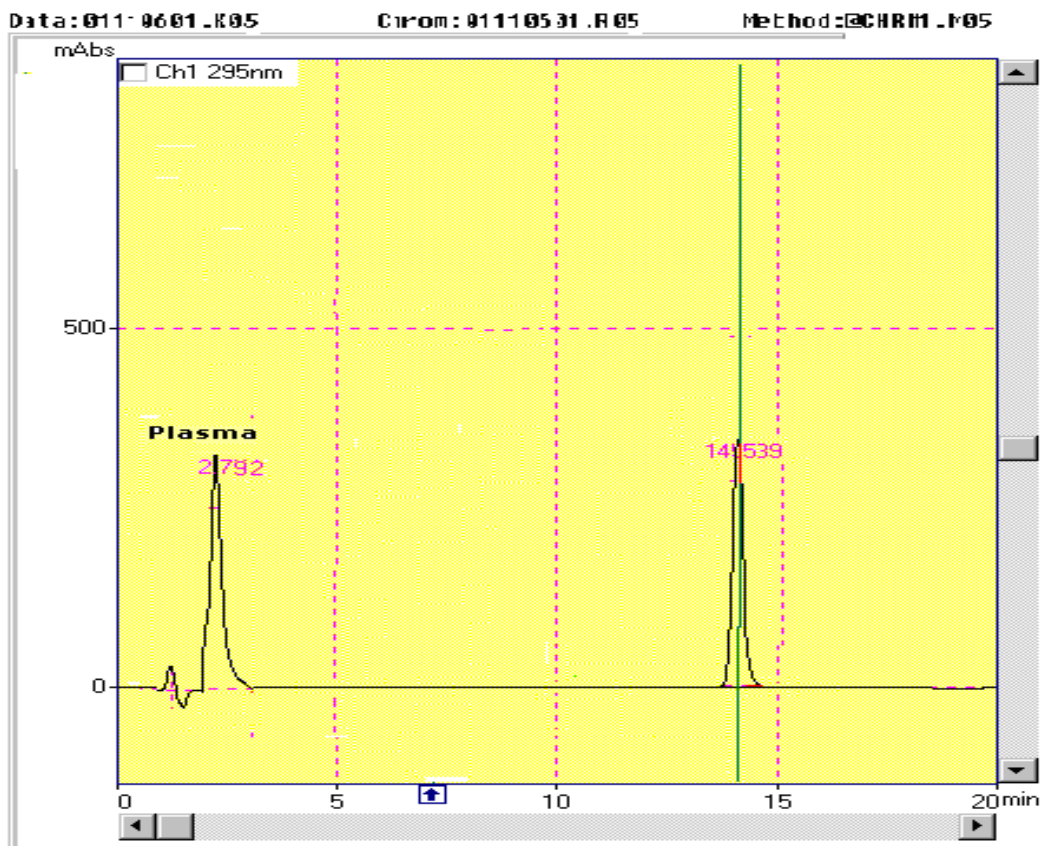


Fig. 4: It shows chromatogram of Lornoxicam (14.53 min) in spiked human plasma (R.T. 2.79 min)

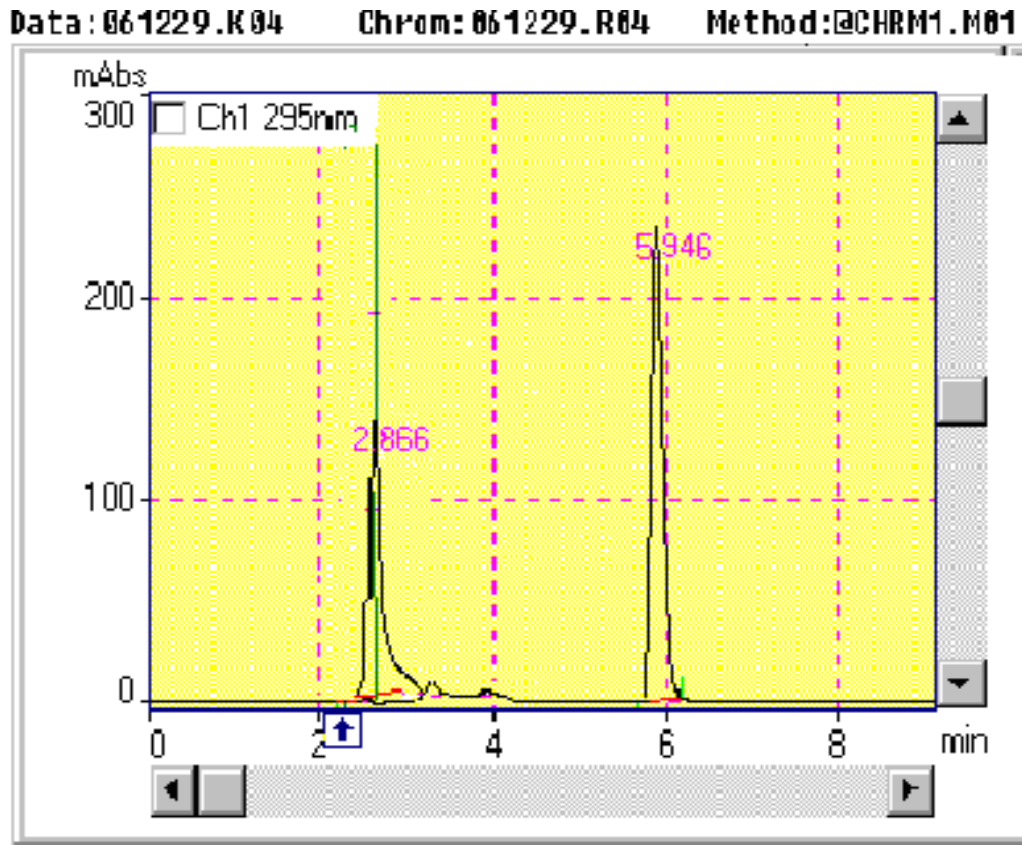


Fig. 5: It shows chromatogram of Thiocolchicoside (5.94 min) in spiked human plasma (R.T. 2.86 min)

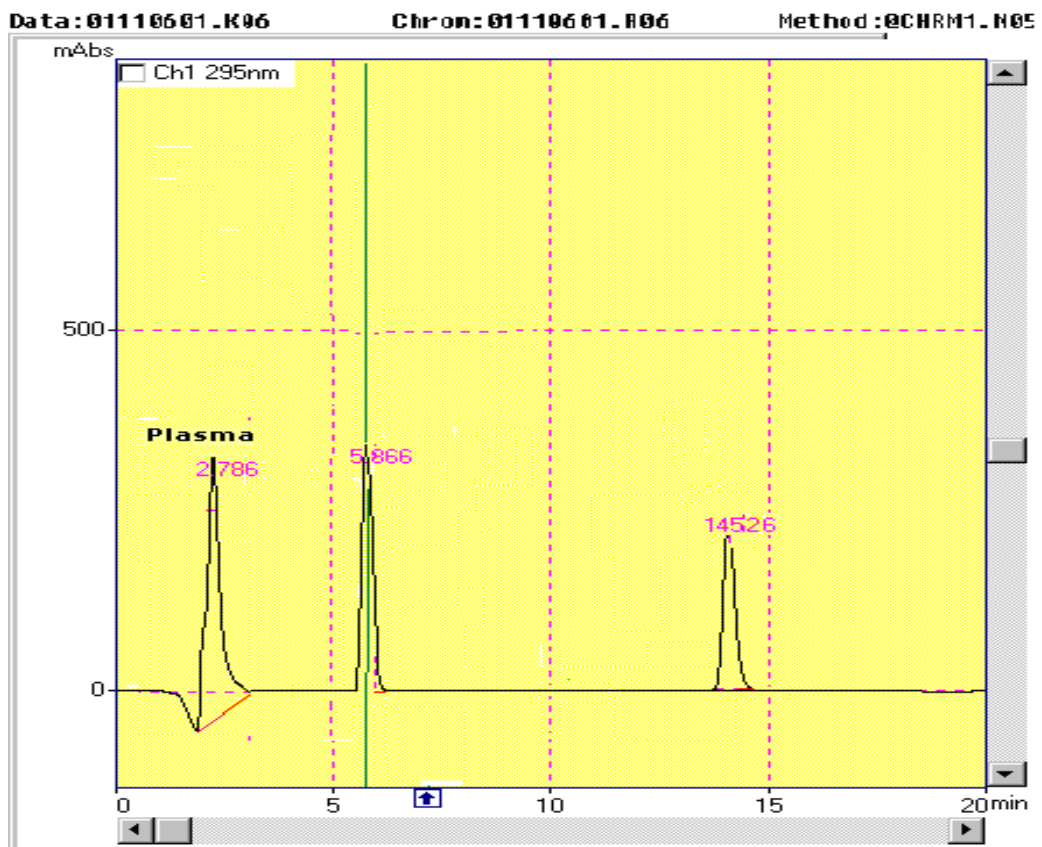


Fig. 6: It shows chromatogram of Thiocolchicoside (5.86 min) and of Lornoxicam (14.52 min) in spiked human plasma (R.T. 2.78 min)

Mobile phase selection: In order to achieve maximum peak responses, symmetrical chromatographic peaks, and resolution of drugs and plasma components, mobile phases containing varying percentages of organic solvents at different pH values were tried. THC, a weak base has pKa value of 10 and LOR 5.5. So the mobile phase having pH 6.8 is selected because in more acidic mobile phase THC is showing peak splitting and in more basic mobile phase LOR is showing peak splitting. Initially Phosphate buffer (pH 6.8) and Acetonitrile (50:50 v/v) was used as mobile phase but it shows merging of plasma peak with THC. So to obtain better resolution organic content was reduced and Phosphate buffer (pH 6.8) and Acetonitrile (70:30 v/v) was tried which gave sharp peaks with good resolution.

Method Validation for human plasma

Selectivity: From the chromatogram of blank plasma as well as chromatogram of Thiocolchicoside and Lornoxicam along with blank plasma it is clear that retention times of plasma (2.79 min) and Thiocolchicoside (5.94 min) as well as Lornoxicam (14.53) are quite different means plasma components are not showing interference with the Thiocolchicoside and Lornoxicam elution.

Limit of detection (LOD) and Limit of quantification (LOQ): LOD for Thiocolchicoside was 33.27 ng/ml and for Lornoxicam 66.2 ng/ml. The LOQ was found to be 100.82 and 200.48 ng/mL for THC and LOR, respectively.

Linearity: Linearity was studied by preparing standard solutions at different concentration levels. The linearity range for THC and LOR were found to be 100- 500 ng/ml and 200 - 1000 ng/ml respectively. The linearity of this method was statistically confirmed. The regression equations for THC and LOR were found to be $y =$

$285.44x - 2671.3$ and $y = 240.26x - 6782.2$ with coefficient of correlation, (r) 0.9983 and 0.9972 respectively which indicate excellent linearity of method.

Accuracy: The % nominal concentration of THC and LOR were found in the range of 85.59 -95.68 % and Coefficient of Variation was 2.06-4.75 (Table No. 3). Since the nominal concentration and % CV is within the range, it shows accuracy of method.

Precision: The method proved to be precise because the % CV for THC and LOR is not more than 6.36 and % mean not less than 85.15 in all the conditions (repeatability as well as intermediate precision, Table No.4). So the acceptance criteria (% CV <15% and % mean $100 \pm 15\%$) were met in all cases.

Recovery: The results of the recovery experiments were satisfactory. The mean absolute recoveries were ranging from 83.43 - 91.57 % for THC and LOR at three different levels of concentration (LQC, MQC, and HQC, Table No.5).

Stability: Stabilities of the samples were determined in various phases of method.

The stability studies include freeze-thaw stability and bench-top stability. All the above stability studies indicate that the samples in various phases were within the acceptance limits.

The concentration of the bench top samples was found to be 92.41 to 95.97 % of the nominal concentration for THC and 92.77-96.96 for LOR, indicating the bench top stability of the analytes. In freeze-thaw stability the concentration of the samples were found to be 93.41 to 96.78 % of the nominal concentration for THC and 95.85 - 96.13 for LOR, indicating the stability of the analytes over freeze-thaw cycles (Table No.6).

Table 3: Table shows Accuracy data for Thiocolchicoside and Lornoxicam

Parameter	Accuracy Data					
	LQC		MQC		HQC	
	THC	LOR	THC	LOR	THC	LOR
Mean	85.64	171.18	277.71	561.99	466.99	956.79
S.D.	4.07	6.49	7.18	18.05	13.07	19.67
% Nominal conc.	85.64	85.59	92.57	93.66	93.40	95.68
% CV	4.75	3.79	2.59	3.21	2.80	2.06

Table 4: Table shows Precision data for Thiocolchicoside and Lornoxicam

Statistical parameter	THC			LOR		
	% Mean	S.D.	% CV	% Mean	S.D.	% CV
Repeatability	85.15	5.41	6.36	92.15	4.02	4.37
Intermediate Precision						
a. Day to day	89.56	3.11	3.47	91.22	4.02	4.41
b. Analyst to Analyst	90.66	3.78	4.17	92.34	2.87	3.11

Table 5: Table shows Recovery data for Thiocolchicoside and Lornoxicam.

Statistical parameter	% Recovery					
	LQC		MQC		HQC	
	THC	LOR	THC	LOR	THC	LOR
% CV	5.26	4.45	3.85	2.37	3.64	3.21
% recovery	89.29	87.85	91.57	83.43	89.21	88.24

Table 6: Table shows Bench Top and freeze-thaw Stability data for Thiocolchicoside and Lornoxicam.

Statistical parameter	Bench Top stability				Freeze-thaw stability			
	LQC conc. (ng/ml)		HQC conc. (ng/ml)		LQC conc. (ng/ml)		HQC conc. (ng/ml)	
	THC	LOR	THC	LOR	THC	LOR	THC	LOR
Mean	73.48	164.98	455.41	926.46	70.51	159.07	449.92	916.88
SD	2.96	2.46	13.91	22.27	4.75	6.44	12.31	14.56
%CV	4.03	1.49	3.05	2.40	6.74	4.05	2.74	1.59
Nominal conc.	79.51	177.84	474.52	955.46	75.48	165.95	464.86	953.79
% nominal	92.41	92.77	95.97	96.96	93.41	95.85	96.78	96.13
% change	7.58	7.23	4.02	3.03	6.59	4.15	3.21	3.87

Method Validation for pharmaceutical dosage form

Results of all the validation parameters are summarized in table no.7.

Linearity: The regression equations for THC and LOR were found to be $y = 301.93x - 2691.8$ and $y = 282.35x - 12378$ with coefficient of correlation, (r) 0.9995 and 0.9957, respectively.

Accuracy: The percent recovery for THC was found to be 99.27 % and for LOR it was 99.25 % with % R.S.D 1.19 and 1.16 respectively.

Precision

Repeatability: The percent R.S.D. for THC was 1.06 and for LOR it was 0.91 which is according to ICH norms.

Intermediate Precision: In analyst to analyst variation % R.S.D. for THC and LOR was 1.85 and 0.82 respectively and in day to day variation it was 1.44 for THC and 1.32 for LOR.

Robustness: The robustness was performed by changing flow rate $\pm 10\%$ and pH ± 0.5 unit, the results were interpreted by statistical analysis by calculating RSD values. All the results were within the acceptance criteria i.e. RSD is not more than 2 %.

Assay of Marketed Formulation

The pharmaceutical preparation of combination of THC and LOR is **Lorchek - MR** (INDOCO Remedies Ltd), labelled to contain 4 mg of THC and 8 mg of LOR.

Table 7: Table shows Validation parameters for Drug Quantitation in pharmaceutical dosage form

Validation Parameters	THC	LOR
Linearity (r²)	0.9995	0.9957
Precision (%RSD found)		
Repeatability	1.06	0.91
Analyst variation	1.85	0.82
Inter day Variation	1.44	1.32
Accuracy by recovery study (%R.S.D. found)	1.19	1.16
Robustness(%RSD)		
pH variation	1.78	0.9
Mobile phase change	1.77	0.71
Tablet Analysis (% R.S.D. found)		
Lorchek - MR	2.47	1.85

Table 8: Table shows Result of analysis of marketed formulation

S. No.	Conc. of drugs claimed in tablet(ng/ml)		Conc. Found (ng/ml)		% Found	
	THC	LOR	THC	LOR	THC	LOR
Rep-1	100	200	91.56	188.32	91.56	94.16
Rep-2	200	400	186.86	384.58	93.43	96.15
Rep-3	300	600	286.54	584.47	95.51	97.41
Rep-4	400	800	387.66	786.63	96.92	98.33
Rep-5	500	1000	484.77	984.77	96.95	98.48
				Mean	94.87	96.90
				S.D.	2.34	1.79
				%R.S.D.	2.47	1.85

The commercially tablet formulation of THC and LOR are present in the ratio of 1:2. Based on this ratio five mixed standards were selected for quantitative analysis, which gave satisfactory results (Table No.8)

CONCLUSION

An accurate, precise and validated isocratic HPLC assay was developed for the simultaneous analysis of Thiocolchicoside and Lornoxicam in human plasma as well as in pharmaceutical dosage form. The method has been validated according to U.S. Food and Drug Administration guidelines for bioanalytical methods and found to be linear, accurate and precise both in upper and lower concentration range with acceptable error and % C.V. values far less than 15. Validation parameters for pharmaceutical dosage form are also found within the limit according to ICH guidelines. As the precision accuracy and robustness are concern the %RSD is less than 2 which is with in range of ICH guidelines.

Since, for preparation of plasma samples, the developed method involves direct estimation (precipitation of plasma protein by organic solvents) which is simple, cheap, accurate and easy in comparison to solid phase extraction or liquid-liquid extraction.

So this HPLC method should be useful for monitoring plasma drug concentrations, and pharmacokinetic studies in patients of musculoskeletal disorders, and for any other studies where it is desirable to measure concentrations of these muscle relaxant and anti inflammatory agents in biological samples as well as it can be used routinely in industry for analysis of drug in pharmaceutical dosage form.

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