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A NEW BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF THIOCOLCHICOSIDE AND PARACETAMOL IN BULK AND PHARMACEUTICAL DOSAGE FORMS IN HUMAN PLASMA BY RP-HPLC METHOD

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ABSTRACT

A simple, rapid, precise and accurate high performance liquid chromatography method was developed for simultaneous determination of THIOCOLCHICOSIDE AND PARACETAMOL in bulk and pharmaceutical dosage forms in human plasma by RP-HPLC method. The analytes were extracted by protein precipitation technique and chromatograph using a mobile phase consisting of acetonitrile, methanol and water (20:20:60) ODS C18 (4.6 x 250mm, 5 μm) column. The flow rate 0.8ml/min and UV detection at 260 nm was employed. The retention time for thiocolchicoside and paracetamol was 4.8 and 5.8min respectively. Linearity for thiocolchicoside and paracetamol was found to be in the range of 2-12ug/mL for both drugs respectively. The method was validated as per the USFDA guidelines and the results were within the acceptance criteria for selectivity, sensitivity, linearity, precision, accuracy, recovery stability of solution, stability of solution in plasma and dilution integrity.

Keywords: Thiocolchicoside and paracetamol, Protein precipitation, Human plasma, RP-HPLC, Simultaneous determination.

INTRODUCTION:

Thiocolchicoside is a muscle relaxant with anti-inflammatory and analgesic effects. It acts as competitive GABAA receptor antagonist and also glycine receptor antagonist with similar potency and nicotinic acetylcholine receptors to a much lesser extent. It has a powerful convulsant activity and should not be used in seizure porn individuals. Its chemical name is *N*-[(7*S*)-3-(β-D-Glucopyranosyloxy)-1, 2-dimethoxy-10-(methylsulfanyl)-9-oxo-5, 6, 7, 9-tetrahydrobenzo[*a*]heptalen-7-yl] acetamide.

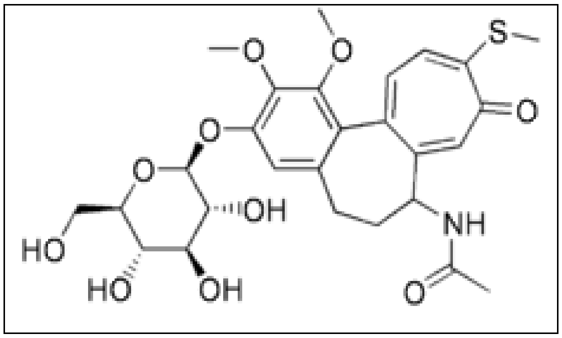


Fig no: - 1 Chemical structure of thiocolchicosie

Mechanism of Action:

Its mode of action includes modulation of chemokine and prostanoid production and inhibition of neutrophil and endothelial cell adhesion molecules by which it interferes with the initiation and amplification of the joint inflammation. It acts as competitive GABAA receptor antagonist and also glycine receptor antagonist with similar potency and nicotinic acetylcholine receptors to a much lesser extent.

Paracetamol is also known as Acetaminophen chemically named as N-acetyl-P-aminophenol, is a widely used over the counter analgesic (Pain reliever) and Antipyretic (fever reducer). Acetaminophen is the name adopted for this pharmacologic agent in the US and Japan.Paracetamol is classified as Mild Analgesic. Its chemical name is N-acetyl-P-aminophenol.

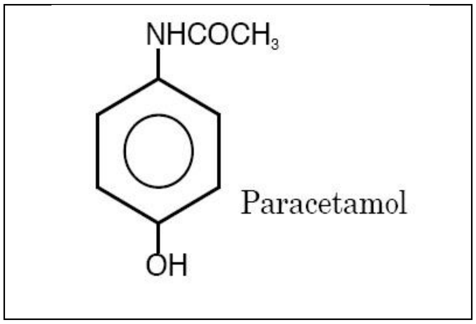


Fig No:2 Chemical Structure of Paracetamol

The main mechanism proposed is the inhibition of cyclo-oxygenase COX), and recent findings suggest that it is highly selective for COX-2.Because of its selectivity for COX-2 it does not significantly inhibit the production of the pro-clotting thromboxanes. While it has analgesic and antipyretic properties comparable to those of aspirin or other NSAIDs, its peripheral anti-inflammatory activity is usually limited by several factors, one of which is the high level of peroxides present in inflammatory lesions. However, in some circumstances, even peripheral anti-inflammatory activity comparable to NSAIDs can be observed.

Instruments and Equipments

- Systronics PC based double beam spectrophotometer 2202 with 1cm matched quartz cells.
- SHIMADZU, Model: Prominence Liquid Chromatograph, SPD- 20A UV – VIS detector. The output signal was monitored and integrated using Lab solutions software. A Symmetry ODS C18 (4.6 x 250mm, 5 μm) column was used for separation.
- Ultra sonicator.
- Cooling centrifuge

Materials and Chemicals

Thiocolchicoside, paracetamol pure forms and dosage forms, Acetonitrile (HPLC grade), Methanol (HPLC grade), water (HPLC grade), human plasma.

METHOD DEVELOPMENT AND VALIDATION:

PREPARATION OF STOCK SOLUTION:

Diluents – Acetonitrile , Methanol and water (20: 20:60)

The stock solutions of Thiocolchicoside and Paracetamol were prepared by dissolving appropriate amount corresponding to 1mg/ml concentrations in the diluents. All the stock solutions were stored at 2 - 8^{0}C. The stock solutions of THC and PARA were further diluted with diluent to give a series of standard mixtures having a final concentration in the range of 20 – 2000ng/mL respectively. A standard 1:1 mixture containing 200ng/mL of THC and PARA were also prepared in the diluent.

Sample preparation:

A simple two step liquid-liquid extraction (LLE) procedure was carried out for the extraction of THC and PARA from plasma samples. To a series of 1mg of drug 1ml of plasma, were added and mixed for 2 min for Proper mixing of drug then add 1ml of HPLC grade methanol centrifuge for 5min at 1000 rpm,4°C for Deprotenation. Separate supernatant liquid filter by using vaccum filteration .then stored in a refrigerator .Take 0.05ml of stock solution in a 10ml volumetric flask make up the drug with diluents up to the mark, mixed for 2min and 2ug/ml sample was injected into HPLC system.

Chromatographic conditions:

Column (Stationary Phase) ODS C18 (4.6 x 250mm, 5 μm, Make: Hypersil)
Mobile Phase Acetonitrile: methanol: water (20:20:60), Flow rate 0.8 (ml/min),
Run time (min) 10, Column temperature (°C) ambient, Volume of injection loop 20ulDetection wavelength (nm) 260, Drug RT (min) 4.8 for THC and 5.8 for PARA.

Method Validation:

1. Selectivity

Selectivity was determined by the analysis of blank and standard samples. The blank sample was tested for interference, and selectivity was ensured at the lower limit of quantification (LLOQ).

Acceptance criteria:-

Precision

Repeatability (Within-run precision):

The repeatability of the method was examined at single intermediate level by injecting the solution consisting of THC and PARA in to the HPLC system for two consecutive days (intra-day and inter-day) respectively.

Acceptance criteria:

Relative Standard Deviation should not be more than 2 %

No interference at retention time of THC and PARA

2. Linearity

For all methods, 6-point calibration curve were prepared on single day. The results obtained were used to calculate the equation of the line by using linear regression by the least square method.

Procedure for calibration curve

The extracted plasma samples, 2-12ug/ml of THC and PARA were injected each time into the column and the corresponding chromatograms were obtained. From these chromatograms retention times and the area under the curve of the drug to that of the reference standard for each dilution was calculated. A relevant calibration curve was constructed with concentration on x-axis and area under the curve on y-axis. The linearity range was found to be from 2-12ug/ml of THC and PARA. The regression equation of the curve was computed.

Acceptance Criteria:

The plot of concentration versus peak area for each drug should be linear with a correlation coefficient (R^2) not less than 0.999.

3. Accuracy of the method:

The accuracy of an analytical method is the closeness of the test results to the true value. It was determined by application of the analytical procedure to recovery studies, where known amount of standard THC and PARA (50%, 100%, and 150%) is spiked. From this, percentage recovery values were calculated.

Acceptance criteria:

Mean recovery should be in between 96– 104%

4. Recovery :

Recovery experiments were performed by comparing the analytical results for extracted samples at three concentrations (LQC, MQC, and HQC) with unextracted standards that represent 100% recovery.

5.Stability Studies:

1. **Freeze and Thaw Stability:**

Analyte stability was determined after three freeze and thaw cycles. Three of LQC and HQC were stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12 to 24 hours under the same conditions. The freeze–thaw cycle were repeated two more times and then analyzed on the third cycle.

2. Short-Term Temperature Stability:

Three aliquots of LQC and HQC were thawed at room temperature and kept at this temperature for 22 hours and analyzed.

3. Long-Term Stability:

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. So, long-term stability was determined by storing three aliquots of

Blank peak:

LQC and HQC under the same conditions as the study samples for 22 days.

4. Stock Solution Stability:

The stability of stock solutions of drug was evaluated at room temperature for 6 hours.

Fig No:- 3 typical HPLC chromatogram:

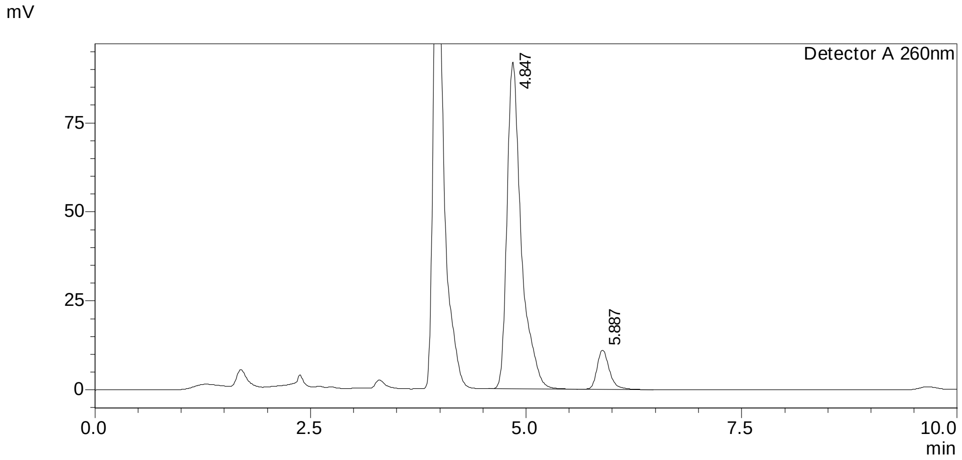
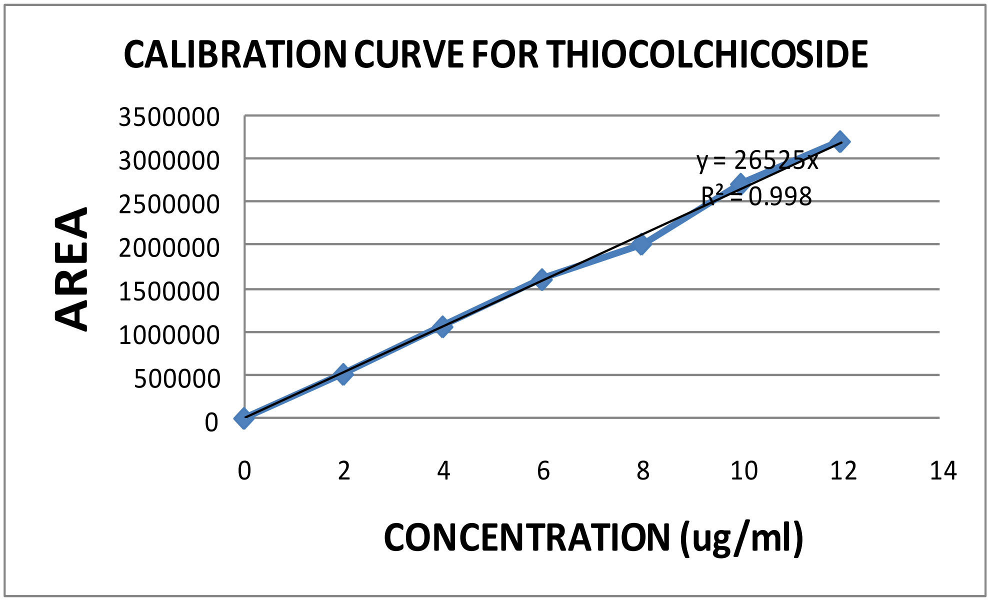


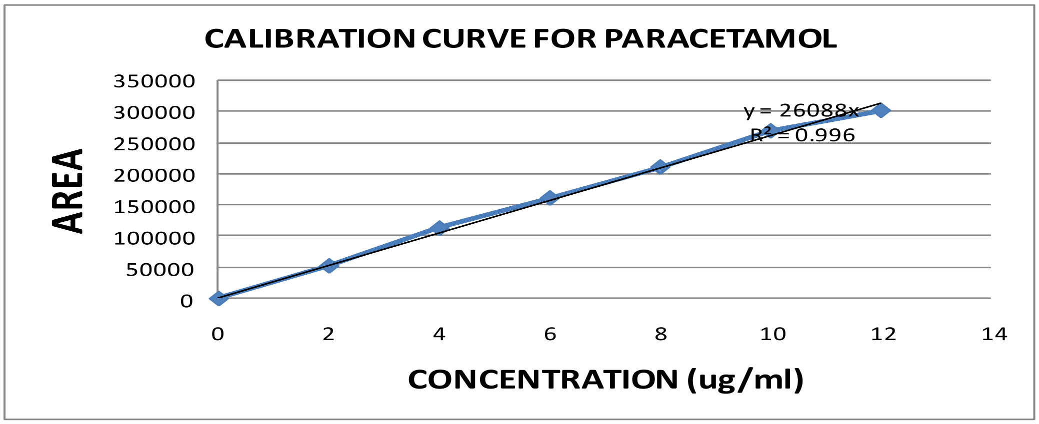
Fig No:- 4 Calibration curve for THIOCOLCHICOSIDE



Tab No; 1 Calibration curve for Thiocolchicoside:

S.No	Linearity Level	Concentration (ug/ml)	Area
1	I	2	509557
2	II	4	1059482
3	III	6	1605843
4	IV	8	2015783
5	V	10	2709732
6	VI	12	3203203
Correlation Coefficient			0.998

Fig No: 5 Calibration curve for Paracetamol:



Tab NO:-2 Linearity data for paracetamol:

S.No	Linearity Level	Concentration (ug/mL)	Area
1	I	2	52300
2	II	4	112582
3	III	6	160916
4	IV	8	210365
5	V	10	268141
6	VI	12	300934
Correlation Coefficient			0.996

Tab No: - 3 Results of the optimized method

S.NO	Parameters	Specifications	Inference	
			THC	PARA
1	Specificity	No interference	No interference	No interference
2	Resolution	NLT 2	4.7	5.8
3	Tailing Factor	NMT 2	1.622	1.207
4	Number of Theoritical Plates	NLT 2000	8942	9432
5	Precision Intra Day (n=6) Inter Day(n=6)	RSD NMT 2%	0.015	0.05
			0.015	0.05
6	Linearity Range	Correlation coefficient NLT 0.999	0.998	0.996
7	Accuracy-Mean Recovery	Range 96-104%	96.7	97.93
8	Recovery %from plasma		98.83	99.2

SUMMARY AND CONCLUSION:

An RP-HPLC method is developed and validated as per ICH guidelines for simultaneous estimation of Thiocolchicoside and paracetamol in human plasma.

The proposed method was found to be rapid, accurate, precise, specific, robust, rugged and economical. The mobile phase is simple to prepare and economical. The sample recoveries in all formulations were in good. This method offers an advantage than reported methods that the drugs can be estimated simultaneously. Thus the method is not time consuming and can be used in clinical studies for the analysis of bioavailability and bioequivalence.

Thus the purpose of the present investigation was successfully achieved

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