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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF DICYCLOMINE HCL AND MEFENAMIC ACID IN DOSAGE FORM

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Abstract:

A reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Dicyclomine Hydrochloride (HCL) and Mefenamic Acid in pharmaceutical dosage forms. The method was optimized for effective separation and quantification of both drugs, ensuring precision, accuracy, and reliability. The chromatographic conditions were optimized using a Waters HPLC system equipped with an auto-sampler and PDA Detector 996 model. The separation was achieved on a Hypersil C18 column (4.6×250 mm, 5 µm) with a mobile phase consisting of acetonitrile and water in a 50:50 (v/v) ratio. The flow rate was set at 1 mL/min, and the detection was performed at 235 nm. The column temperature was maintained at 35°C, and the injection volume was 10 µL. The total run time for the analysis was 10 minutes, allowing for efficient separation and accurate estimation of both drugs within a short period. The developed method was validated according to ICH guidelines for parameters such as linearity, precision, accuracy, specificity, robustness, and limit of detection/quantification. The method showed good linearity over a concentration range, and the validation results confirmed the method's suitability for routine quality control analysis of Dicyclomine HCL and Mefenamic Acid in their combined dosage forms.

Keywords: *Dicyclomine Hydrochloride (HCL) and Mefenamic, RP-HPLC, Simultaneous Estimation, Pharmaceutical Dosage Forms, Waters HPLC.*

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1. INTRODUCTION

0 Introduction to HPLC:

High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today.1In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, and production.2 HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes is in synthetic procedures or scale up, evaluating new formulations and carrying out quality control / assurance of the final drug products.3

The Goal of HPLC method is to try & separate, quantify the main drug, any reaction impurities, all available synthetic intermediates and any degradants. 4High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. HPLC is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability. 5 HPLC principle is the solution of sample is injected into a column of porous material (stationary phase) and liquid phase (mobile phase) is pumped at higher pressure through the column. The principle of separation followed is the adsorption of solute on stationary phase based on its affinity towards stationary phase. (Figure-1) The technique of HPLC has following features.6

- High resolution
- Small diameter, Stainless steel, Glass column
- Rapid analysis
- Relatively higher mobile phase pressure
- Controlled flow rate of mobile phase

HPLC Method Development:

Methods are developed for new products when no official methods are available. Alternate methods for existing (Non-Pharmacopoeial) products are to reduce the cost and time for better precision and ruggedness. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available. The goal of the HPLC-method is to try & separate, quantify the main active drug, any reaction impurities, all available synthetic inter-mediates and any degradants.7

Steps involved in Method development are. 6,7

- Understanding the Physicochemical properties of drug molecule.
- Selection of chromatographic conditions.
- Developing the approach of analysis.
- Sample preparation
- Method optimization
- Method validation (figure-2)

Understanding the physicochemical properties of drug molecules:

Physicochemical properties of a drug molecule play an important role in method development. For Method development one has to study the physical properties like solubility, polarity, pKa and pH of the drug molecule. Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase. 6 The solubility of molecules can be explained on the basis of the polarity of molecules. Polar, e.g. water, and nonpolar, e.g. benzene, solvents do not mix. In general, like dissolves like i.e., materials with similar polarity are soluble in each other. Selection of diluents is based on the solubility of analyte. The acidity or basicity of a substance is defined most typically by the pH value. Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC.7

Selection of chromatographic conditions

During initial method development, a set of initial conditions (detector, column, mobile phase) is selected to obtain the first “scouting” chromatograms of the sample. In most cases, these are based on reversed-phase separations on a C18 column with UV detection. A decision on developing either an isocratic or a gradient method should be made at this point.

Selection of Column:

A column is of course, the starting and central piece of a chromatograph. A appropriately selected column can produce a good chromatographic separation which provides an accurate and reliable analysis. An improperly used column can often generate confusion, inadequate, and poor separations which can lead to results that are invalid or complex to interpret.9The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Choosing the best column for application requires consideration of stationary phase chemistry, retention capacity, particle size, and column dimensions. The three main components of an HPLC column are the hardware, the matrix, and the stationary phase.

There are several types of matrices for support of the stationary phase, including silica, polymers, alumina, and zirconium. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily

derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One short coming of a silica solid support is that it will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high pH. The nature, shape and particle size of the silica support effects separation. Smaller particle results in a greater number of theoretical plates, or increased. The nature of the stationary phase will determine whether a column can be used for normal phase or reverse phase chromatography.

Normal phase chromatography utilizes a polar stationary phase and a non-polar mobile phase. Generally, more polar compounds elute later than non-polar compounds. Commonly used reverse phase columns and their uses are listed below. Propyl (C3), Butyl (C4), and Pentyl (C5) phases are useful for ion-pairing chromatography (C4) and peptides with hydrophobic residues, and other large molecules. C3–C5 columns generally retain non-polar solutes more poorly when compared to C8 or C18 phases. Examples include Zorbax SB-C3, YMC-Pack C4, and Luna C5. These columns are generally less stable to hydrolysis than columns with longer alkyl chains. Octyl (C8, MOS) phases have wide applicability. This phase is less retentive than the C18 phases, but is still quite useful for pharmaceuticals, nucleosides, and steroids.10Selection of the stationary phase/column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high performance column. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible. The separation selectivity for certain components vary between the columns of different manufacturer as well as between column production batches from the same manufacturer. Column dimensions, silica substrate properties and bonded stationary phase characteristics are the main ones. The use of silica-based packing is favored in most of the present HPLC columns due to several physical characteristics.6

Selection of Chromatographic mode: chromatographic modes based on the analyte's molecular weight and polarity. All case studies will focus on reversed-phase chromatography (RPC), the most common mode for small organic molecules. Ionizable compounds (acids and bases) are often separated by RPC with buffered mobile phases (to keep the analytes in a non-ionized state) or with ion-pairing reagents.8

Optimization of Mobile phase:

Buffer Selection: Different buffers such as potassium phosphate, sodium phosphate and acetate were evaluated for system suitability parameters and overall chromatographic performance.

Effect of pH.:- If analytes are ionizable, the proper mobile-phase pH must be chosen based on the analyte p*Ka* so the target analyte is in one predominate ionization state, ionized or neutral. Alteration of the mobile-phase pH is one of the greatest tools in the "chromatographer's toolbox" allowing simultaneous change in retention and selectivity between critical pair of components.12

- **Effect of organic modifier:** -Selection of the organic modifier type is relatively simple in reverse phase HPLC, The usual choice is between Acetonitrile and methanol (rarely THF). Gradient elution is usually employed with complex multicomponent samples since it may not be possible to get all components eluted between k *(*retention factor*)* 1 and 10 using a single solvent strength under isocratic conditions. 12

Selection of detector and wavelength:

After the chromatographic separation, the analyte of interest is detected by using suitable detectors. Some commercial detectors used in LC are: ultraviolet (UV) detectors, fluorescence detectors, electrochemical detectors, refractive index (RI) detectors and mass spectrometry (MS) detectors. The choice of detector depends on the sample and the purpose of the analysis. In case of multicomponent analysis the absorption spectra may have been shifted to longer or shorter wavelengths compared to the parent compound. Therefore the UV spectra of target analyte and impurities must be taken and overlaid with each other, and the spectra should be normalized due to different amounts present in the mixture. A wavelength must be chosen such that adequate response is for most of the analytes can be obtained.[12,13]

MATERIALS AND METHODS:

Dicyclomine HCl- Provided by Sura Pharma labs, Mefenamic Acid-Provided by Sura Pharma labs, Water and Methanol for HPLC-LICHROSOLV (MERCK), Acetonitrile for HPLC- Merck, Triethylamine- Sura labs

HPLC METHOD DEVELOPMENT:

TRAILS

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Dicyclomine HCl and Mefenamic Acid working standard into a

10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.
Further pipette 0.2ml of the Dicyclomine HCl and o.4ml of the Mefenamic Acid stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:
Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:
Initially the mobile phase tried was Methanol: Water and Methanol: TEA Buffer with varying proportions. Finally, the mobile phase was optimized to Acetonitrile: Water in proportion 65:35 v/v respectively.

Optimization of Column:
The method was performed with various columns like Symmetry and Phenomenex. Gemini C18 (4.6×150mm, 5µ) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used	:	Waters HPLC with auto sampler and PDA Detector 996 model.
Temperature	:	35ºC
Column (4.6×250mm) 5µ	:	Hypersil C18
Mobile phase Water (50:50v/v)	:	Acetonitrile:
Flow rate	:	1ml/min
Wavelength	:	235 nm
Injection volume	:	10 µl
Run time	:	10 min

METHOD VALIDATION
PREPARATION OF MOBILE PHASE:
Preparation of mobile phase:
Accurately measured 500 ml (50%) of Water, 500ml of Acetonitrile (50%) were mixed and degassed in digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:
The Mobile phase was used as the diluent.

RESULTS AND DISCUSSION:

Optimized Chromatogram (Standard)

Mobile phase ratio	: Acetonitrile: Water (50:50v/v)
Column	: Hypersil C18 (4.6×250mm) 5µ
Column temperature	: 40ºC
Wavelength	: 235nm
Flow rate	: 0.9ml/min
Injection volume	: 10µl
Run time	: 8minutes

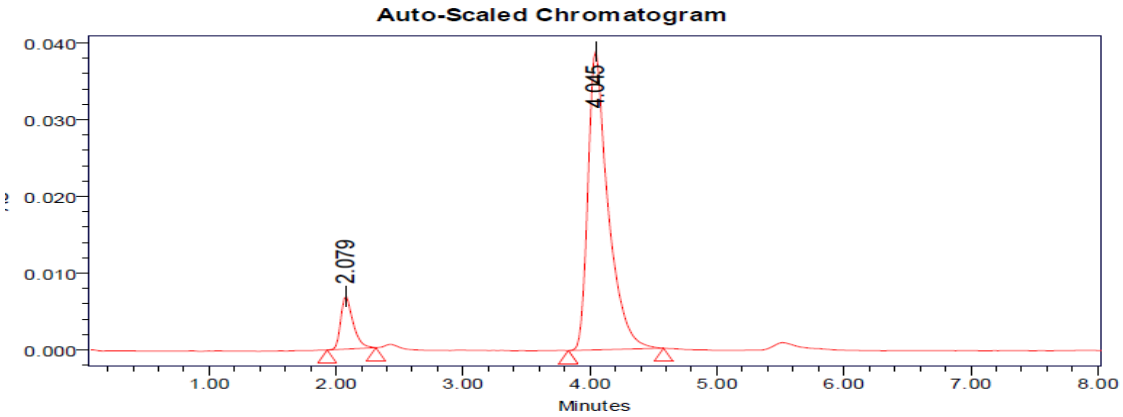

Fig: Optimized Chromatogram (Standard)

Table : Optimized Chromatogram (Standard)

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Dicyclomine HCl	2.079	46168	6841	1.33	4251
2	Mefenamic Acid	4.045	429069	38885	1.59	5224

Optimized Chromatogram (Sample)

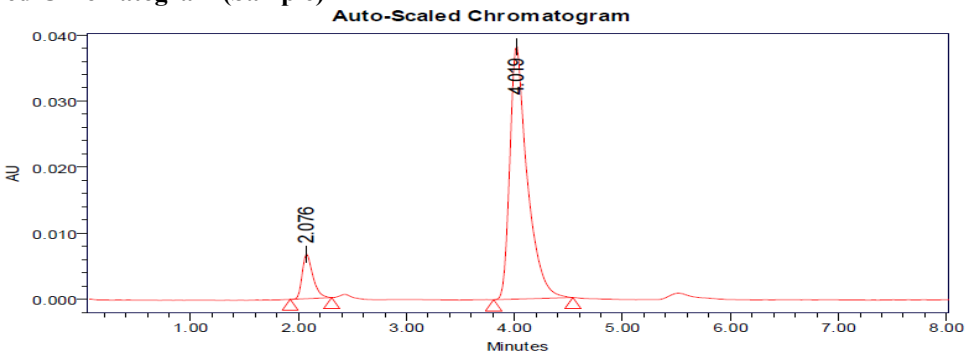

Fig: Optimized Chromatogram (Sample)

Table : Optimized Chromatogram (Sample)

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Dicyclomine HCl	2.076	46150	6766	1.36	5152
2	Mefenamic Acid	4.019	427826	38246	1.58	6071

Acceptance Criteria:

- Theoretical plates must be not less than 2000.
- Tailing factor must be not less than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

Assay (Standard):

Table No. 20: Peak results for assay standard of Dicyclomine HCl

S.No.	Peak Name	RT	Area	Height (µV)	USP Plate	USP Tailing
1	Dicyclomine HCl	2.078	49569	6811	6945	1.51
2	Dicyclomine HCl	2.080	49649	6999	6149	1.57
3	Dicyclomine HCl	2.078	49731	6972	6473	1.49
4	Dicyclomine HCl	2.079	49479	6971	6190	1.49
5	Dicyclomine HCl	2.082	49684	6841	6294	1.49
Mean			49607			
Std. Dev.			107.963			
% RSD			0.217637			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is suitable.

Table : Peak results for assay standard of Mefenamic Acid

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Mefenamic Acid	4.041	423328	44147	7672	1.35
2	Mefenamic Acid	4.033	423805	44538	7786	1.13
3	Mefenamic Acid	4.050	423229	44964	5772	1.34
4	Mefenamic Acid	4.045	423876	44959	5191	1.35
5	Mefenamic Acid	4.032	423575	38885	5137	1.35
Mean			423559.5			
Std. Dev.			328.2606			
% RSD			0.0775			

Acceptance Criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is suitable.

Assay (Sample):

Table : Peak results for Assay sample of Dicyclomine HCl

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Dicyclomine HCl	2.078	46684	6918	1.34	5217	1
2	Dicyclomine HCl	2.079	46168	6841	1.33	5251	2
3	Dicyclomine HCl	2.077	46088	6851	1.37	7127	3

Table : Peak results for Assay sample of Mefenamic Acid

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Mefenamic Acid	4.050	430575	39127	1.60	6197
2	Mefenamic Acid	4.045	429069	38885	1.59	6224
3	Mefenamic Acid	4.037	429543	38892	1.58	8203

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Dicyclomine HCl and Mefenamic Acid in pharmaceutical dosage form was found to be 98.2%

LINEARITY:

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY FOR DICYCLOMINE HCL:

Concentration µg/ml	Average Peak Area
20	15065
40	31009
60	46166
80	60569
100	76862

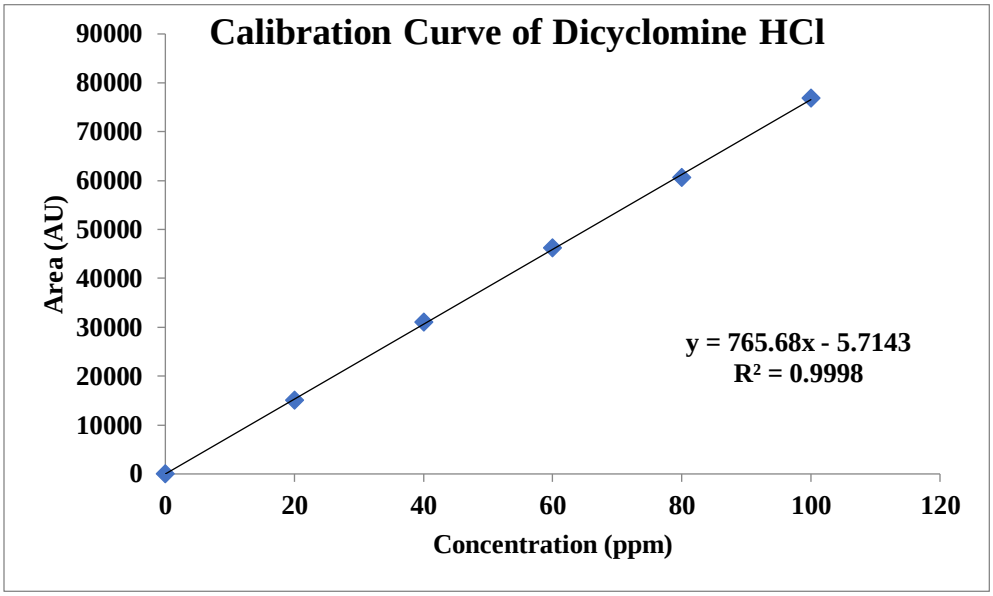

Fig: Chromatogram showing linearity level

MEFENAMIC ACID:

Concentration µg/ml	Average Peak Area
40	131289
80	284775
120	427559
160	555861
200	712514

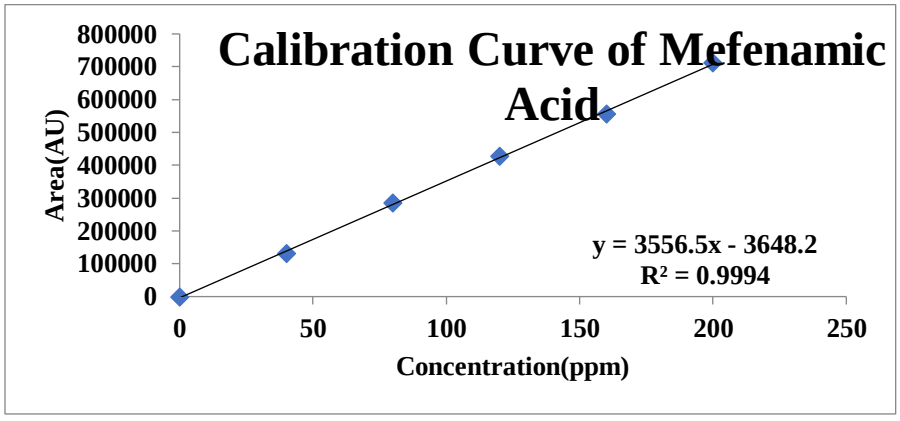

Fig: Chromatogram showing linearity level

Precision:
REPEATABILITY
Table: Results of repeatability for Dicyclomine HCl:

S. No.	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Dicyclomine HCl	2.077	46054	6784	4208	1.32
2	Dicyclomine HCl	2.076	46803	6867	6088	1.34
3	Dicyclomine HCl	2.076	46150	6766	4152	1.36
4	Dicyclomine HCl	2.077	46056	6715	4184	1.32
5	Dicyclomine HCl	2.074	46247	6746	4065	1.33
Mean			46262			
Std.dev			312.7099			
%RSD			0.675954			

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table : Results of repeatability for Mefenamic Acid:

S. No	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Mefenamic Acid	4.031	427962	38634	5158	1.57
2	Mefenamic Acid	4.024	429623	38673	5092	1.58
3	Mefenamic Acid	4.019	427826	38246	5071	1.58
4	Mefenamic Acid	4.016	427829	38310	5046	1.58
5	Mefenamic Acid	4.014	429559	38181	5036	1.58
Mean			428559.8			
Std.dev			943.2246			
%RSD			0.220092			

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Intermediate precision:
Day 1:

Table : Results of Intermediate precision day1 for Dicyclomine HCl

S.No			Area	Height (µV)		
1	Dicyclomine HCl	2.075	46204	6673	5117	1.33
2	Dicyclomine HCl	2.074	46300	6735	5043	1.36
3	Dicyclomine HCl	2.075	46259	6652	5087	1.28
4	Dicyclomine HCl	2.075	46223	6667	5134	1.31
5	Dicyclomine HCl	2.075	46205	6674	5151	1.32
6	Dicyclomine HCl	2.074	46189	6703	5157	1.33
Mean			46230			
Std. Dev.			41.88556			
% RSD			0.090603			

Acceptance criteria:
%RSD of Six different sample solutions should not more than 2.

Table : Results of Intermediate precision day1 for Mefenamic Acid

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Mefenamic Acid	4.013	428922	38004	7038	1.58
2	Mefenamic Acid	4.011	428524	37935	7999	1.57
3	Mefenamic Acid	4.010	427239	37850	7003	1.57
4	Mefenamic Acid	4.008	427667	37780	7982	1.57
5	Mefenamic Acid	4.006	427826	37824	7983	1.57
6	Mefenamic Acid	4.006	427093	37970	7042	1.58
Mean			427878.5			
Std. Dev.			718.1952			
% RSD			0.16785			

Acceptance criteria:

- %RSD of Six different sample solutions should not more than

DAY 2:

Table : Results of Intermediate precision Day 2 for Dicyclomine HCl

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Dicyclomine HCl	2.076	46803	6867	5149	1.57
2	Dicyclomine HCl	2.076	46056	6715	5190	1.13
3	Dicyclomine HCl	2.077	46252	6652	6088	1.58
4	Dicyclomine HCl	2.075	46205	6674	5184	1.58
5	Dicyclomine HCl	2.075	46940	7249	5087	1.57
6	Dicyclomine HCl	2.072	46727	6983	5151	1.57
Mean			46497.17			
Std. Dev.			369.4739			
% RSD			0.794616			

Acceptance criteria:
%RSD of Six different sample solutions should not more than 2 Table:

Table : Results of Intermediate precision Day 2 for Mefenamic Acid

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Mefenamic Acid	4.024	429623	38673	6789	1.49
2	Mefenamic Acid	4.024	427829	38310	5772	1.34
3	Mefenamic Acid	4.016	427263	37850	5092	1.32
4	Mefenamic Acid	4.010	427826	37824	6046	1.28
5	Mefenamic Acid	4.006	421284	40752	6003	1.32
6	Mefenamic Acid	4.008	421832	40281	6983	1.33
Mean			425942.8			
Std. Dev.			3492.681			
% RSD			0.819988			

Acceptance criteria:

- %RSD of Six different sample solutions should not more than 2.

ACCURACY:

Table : The accuracy results for Dicyclomine HCl

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	22938.33	30	29.9655	99.88	100.166
100%	45426	60	59.33511	98.89	
150%	70096.67	90	91.55572	101.7285	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Table : The accuracy results for Mefenamic Acid

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	209357	60	59.8	99%	99%
100%	420697.7	120	119.8	99%	
150%	631550.7	180	179.8	99%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Robustness

Table : Results for Robustness -Dicyclomine HCl

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 0.9mL/min	46168	2.079	4251	1.33
Less Flow rate of 0.8mL/min	51177	2.29	5269	1.38
More Flow rate of 1.0mL/min	42190	1.890	5126	1.32
Less organic phase (about 5 % decrease in organic phase)	42402	1.885	5126	1.19
More organic phase (about 5 % Increase in organic phase)	42112	1.908	5854	1.36

Table : Results for Robustness-Mefenamic Acid

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 0.9mL/min	429069	4.045	5224	1.59
Less Flow rate of 0.8mL/min	472673	4.450	6328	1.58
More Flow rate of 1.0mL/min	392497	3.660	6217	1.54
Less organic phase (about 5 % decrease in organic phase)	391379	4.251	6996	1.61
More organic phase (about 5 % Increase in organic phase)	391703	3.239	6120	1.50

Acceptance criteria:
The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

SUMMARY AND CONCLUSION:

Summary

The developed RP-HPLC method for the simultaneous estimation of Dicyclomine Hydrochloride (HCL) and Mefenamic Acid in pharmaceutical dosage forms offers an effective and reliable analytical approach. The method was optimized using Waters HPLC with a PDA Detector 996 model, employing a Hypersil C18 column (4.6×250 mm, 5 µm) and a mobile phase of acetonitrile and water in a 50:50 (v/v) ratio. Key parameters such as flow rate, injection volume, and wavelength were carefully selected to achieve optimal separation and quantification. The method was validated following ICH guidelines for parameters such as linearity, accuracy, precision, specificity, and robustness, ensuring its reliability for routine analysis. The chromatographic run time was just 10 minutes, making the method time-efficient for quality control purposes.

CONCLUSION:

The RP-HPLC method developed for the simultaneous estimation of Dicyclomine HCL and Mefenamic Acid in dosage forms is validated as a precise, accurate, and efficient technique for routine pharmaceutical analysis. The optimized chromatographic conditions provide clear separation of both drugs, ensuring accurate quantification in combination formulations. The method's robustness and adherence to ICH validation guidelines make it suitable for use in quality control laboratories, offering a valuable tool for the pharmaceutical industry to ensure the quality and consistency of Dicyclomine HCL and Mefenamic Acid formulations.

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