DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR CONTENT DETERMINATION OF MELOXICAM IN INJECTIONS

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Abstract: The principal aim of this paper is to establish and validate a rapid, simple, and economical approach that employs high-pressure liquid chromatography, capable of routinely assessing the content of Meloxicam present in injections. To accomplish this goal, it is necessary to meet the requirements outlined by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, as well as the specifications delineated in the contemporary editions of the internationally recognized pharmacopeias that pertain to the design and validation of analytical methodologies. The analytical methodology was performed with the utilization of a high-performance liquid chromatography system, Waters Alliance (Waters corporation, USA), consisting of a quadrupole pump, an e-2695 separation module, and an automatic sampler. The optimization of the detection wavelength was accomplished using the Waters 2489 UV/Vis detector and Empower 3 software was employed for data processing. Separation was achieved via the deployment of a LiChrospher 100, RP-18 (5 μm) column. The mobile phase used in the study consisted of a combination of Acetonitrile and ultrapure water, in a 60:40 ratio, respectively. The pH of the water component was subsequently adjusted to 3.1 with the addition of glacial acetic acid. This reversed-phase column approach, using an isocratic method, was then utilized for the successful validation of the analytical method. According to the obtained results, the developed analytical method exhibits accuracy and precision under consistent conditions over a limited period and on a single sample, as well as precision when conducted in the same laboratory on the same day by two analysts. Furthermore, the method is specific, linear across the range, and robust against variations in the ratio of the mobile phase components, the pH of the water in the mobile phase, and the flow rate. These findings support the utility and reliability of the developed methodology for the routine determination of Meloxicam content in injections. In conclusion, the reversed-phase column approach using an isocratic method proved to be a successful and robust analytical method for the determination of Meloxicam content in injections. This fast, simple, and cost-effective alternative offers a promising solution for the analysis of other related drugs with similar chemical properties. Furthermore, the simplicity and ease of application of this method offer significant advantages, as it does not require any special preparation of the working environment or prior training of the analyst. Thus, this method represents a valuable contribution to the field of pharmaceutical analysis, and it may facilitate the quality control of Meloxicam-containing products. Overall, this study provides a foundation for further development and optimization of analytical methods for the analysis of other drugs with similar properties, leading to better quality control and improved patient safety.
Keywords: Meloxicam, injections, content determination, validation, quality control.

1. INTRODUCTION

Meloxicam, a medication that goes by the chemical name 4-Hydroxy-2-methyl-N-(5-methylthiazol-2-yl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide, is a non-steroidal anti-inflammatory drug commonly prescribed to alleviate pain and treat inflammatory conditions. Meloxicam inhibits the activity of enzymes known as

cyclooxygenase-1 and cyclooxygenase-2 and is typically utilized in the management of rheumatoid arthritis, acute exacerbations of osteoarthritis, ankylosing spondylitis, and juvenile idiopathic arthritis (Khalil & Aldosari, 2020).

Method validation is a systematic process that involves various evaluations aimed at verifying that an analytical test system is fit for its intended purpose and can generate reliable and valid analytical data. A validation study entails testing several characteristics of a method to ascertain that it can consistently produce useful and accurate results when used in routine testing. To effectively assess method parameters, the validation test should encompass standard testing conditions, including product excipients. Thus, a method validation study is a product specific (Stauffer, 2018).

Although internationally recognized pharmacopeias have established validated analytical methods for quality control, there is a need to develop and validate new analytical methods that can be utilized in all analytical laboratories. These methods should be fast, simple, and cost-effective to facilitate their widespread adoption. In this context, an analytical method for content determination of Meloxicam in injections was validated at the laboratory of the Department of Drug Quality Control and Pharmaceutical Chemistry at the Faculty of Medical Sciences at Goce Delcev University, Stip. The method was validated in accordance with the guidelines and suggestions put forth by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and the contemporary editions of the internationally recognized pharmacopeias, as well as relevant scientific literature. In accordance with the ICH Guideline Q2(R1), accuracy, precision, specificity, linearity, and range are mandatory parameters for the validation of an analytical method intended for content determination. Although not mandatory, it is advisable to include the parameter of robustness at a certain stage of the analytical method's development process, in order to evaluate the analytical method's ability to remain consistent under the influence of small yet significant variations of the method's parameters.

The accuracy of an analytical procedure is the closeness of the test results obtained by that procedure to the true value (USP 44-NF 39, 2021). The process of determining accuracy requires a minimum of nine determinations, which must be performed at three different concentration levels within the specified range of the analytical method. This is achieved by adding known quantities of a standard solution to a sample solution, a process known as spiking. The accuracy of the method is then expressed as recovery, which is calculated as a percentage of the ratio between the obtained value and the theoretical value. The percent recovery of the spiked solutions should be within 100 ± 2.0% for the average of each set of the three concentration levels, and each individual sample recovery should lie within the range of 98 – 102% (Bliesner, 2006).

The precision of an analytical method refers to the extent of closeness between individual results when the method is carried out by repeatedly sampling the same homogeneous sample under the prescribed conditions. In general, precision can be assessed at different levels, including repeatability, intermediate precision, and reproducibility, but in the case of an analytical method for content determination, it is evaluated only at two levels – repeatability and intermediate precision (ICH Guideline Q2(R1), 2005).

Repeatability represents precision under the same conditions, within a short period, and on the same sample, while intermediate precision reflects the variation in results obtained when working in the same laboratory but under different conditions, such as different days, analysts, and instruments (ICH Guideline Q2(R1), 2005). In this particular case, the intermediate precision of the analytical method was evaluated within one day in the same laboratory by two analysts. When assessing repeatability, the fundamental acceptance criterion is that the relative standard deviation (RSD), obtained from the results of six consecutive injections of the same sample for content determination, should not exceed 2.0%. For intermediate precision, three acceptance criteria have been established. Firstly, the RSD obtained from the results of content determination by a single analyst should not be greater than 2.0%. Secondly, the RSD obtained by combining the results of content determination by two analysts should not be greater than 3.0% (Bliesner, 2006). Finally, the ratio of variances calculated using the F-test should be lower than the critical value (Harvey, 2000).

For the comparison of two variants, a special statistical test, denoted as the F-test, is used to determine whether the difference between them is too large to be attributed to random errors. The F-test is calculated as the ratio between the two variances, with the larger variance as the numerator and the smaller variance as the denominator. Based on the calculated value, the validity of the null hypothesis is determined, and is either accepted or rejected. To perform the F-test, the level of significance at which the analysis is conducted needs to be defined. As is typically the case, an α level of significance of 0.05 was employed in this study, indicating that the likelihood of the tested data arising under the null hypothesis is less than 5%. The critical value (F) represents the largest value of the F-test that can be attributed to random errors at a given level of significance and a given number of degrees of freedom in the numerator and the denominator (Harvey, 2000). If the intermediate precision of an analytical method is determined by two analysts, the degrees of freedom in the numerator can be calculated as n–1, where n represents the number of analysts. In this case, the number of degrees of freedom in the numerator is 1. Given that both analysts analyzed six

samples, each of which was injected twice consecutively, it can be inferred that one analyst conducted 12 analyses, while both analysts together conducted 24 analyses. Therefore, the degrees of freedom in the denominator can be calculated by subtracting the number of analysts from the total number of analyses. In this specific case, the degrees of freedom in the denominator are 22.

Specificity refers to the ability of a method to measure a particular analyte accurately and precisely within a complex mixture, while minimizing any interference from other components in the mixture. The acceptance criterion for specificity of an analytical method involves verifying that the analyte peak is well-resolved from any other peaks in the chromatogram, and that there is no significant interference or overlap in the elution zone of the analyte (Patil et al., 2019).

Linearity pertains to the capacity of an analytical method to generate outcomes that are directly proportional to the concentration of the analyte and should be assessed and validated within the range of the method. It is typically established at a minimum of five distinct concentrations, and the resultant linearity outcomes are subjected to statistical evaluation through regression line calculation using the least squared method (ICH Guideline Q2(R1), 2005). The correlation coefficient (r) and coefficient of determination (R^2) are commonly used mathematical measures of linearity (Miller & Miller, 2010). In the case of an analytical method for content determination, the acceptance criterion is the attainment of a R^2 of ≥ 0.995 (ORA Laboratory Manual Volume II, 2020).

The range of an analytical method is the span between the highest and lowest concentration levels, including these levels, that allows for acceptable precision, accuracy, and linearity. For an analytical method used for content determination, the minimum required range should fall between 80% and 120% of the working concentration (ICH Guideline Q2(R1), 2005).

The robustness of an analytical method refers to its ability to withstand intentional and minor variations in method parameters, thereby demonstrating reliability during routine use (ICH Guideline Q2(R1), 2005). To evaluate the robustness of this analytical method, three types of variations were monitored. The first involved altering the ratio of mobile phase components by ± 5% in relation to the major component. The second involved varying the flow rate by ± 0.2 mL/min relative to the working flow rate. The final variation consisted of modifying the pH value of the ultrapure water in the mobile phase composition by ± 0.2 pH units relative to the working pH value. The robustness of the analytical method was assessed through an analysis of the chromatographic peaks derived from standard and sample solutions. This assessment focused on the capacity factor (K'), symmetry factor (A_S), number of theoretical plates (N). The capacity factor is a dimensionless quantity that measures the extent to which a substance is distributed or adsorbed in the stationary phase from the mobile phase (Watson, 2012). The acceptable range for the capacity factor is between 2 and 10 (Bliesner, 2006). The symmetry factor measures the degree of symmetry of a chromatographic peak, and the acceptable range for the symmetry factor is between 0.8 and 1.8 for analytical methods used for content determination (Ph. Eur. 11.0, 2023). The number of theoretical plates is a measure of the column efficiency and its ability to produce narrow and sharp peaks (Watson, 2012). The acceptance criterion for the number of theoretical plates is ≥ 1000 for analytical methods used for content determination. Finally, the injections of the standard and sample solutions should have a RSD of $\leq 2.0\%$ for each specified parameter (capacity factor, symmetry factor, number of theoretical plates) (Bliesner, 2006).

2. MATERIALS AND METHODS

The method was validated using a high-performance liquid chromatography (HPLC) system, specifically the Waters Alliance system. The system included a quadrupole pump, an e-2695 separation module, and an automatic sampler, with a Waters 2489 UV/Vis detector used for detection and Empower 3 software for data processing. The separation process was carried out using LiChrospher 100, RP-18 (5 μm) column. The analysis was performed at room temperature, which was maintained at 25 °C, with a flow rate of 1.5 mL/min, and a wavelength of 360 nm. The injection volume was set at 10 μL, and the run time was set for 10 minutes.

Meloxicam (99.0%) was used as the reference standard. The tested preparation was Melox 15 mg/1,5 mL solution for injection. Methanol (HPLC, ≥ 99.9%, Sigma Aldrich) was the solvent for both the standard and sample solutions. A mobile phase of a 60:40 (v/v) mixture of Acetonitrile (HPLC, ROTISOLV) and ultrapure water adjusted to pH 3.1 using glacial acetic acid was used.

For the standard solution, about 10.0 mg of Meloxicam reference standard is mixed with half the volume of Methanol in a 50.0 mL volumetric flask, and the mixture is placed in an ultrasonic bath for 20 minutes. The flask is then filled to the mark with the same solvent to create 0.2 mg/mL working concentration. For the sample solution, 1.0 mL of Melox 15 mg/1.5 mL solution is mixed with Methanol in a 50.0 mL volumetric flask and placed in an ultrasonic bath for 20 minutes to create a 0.2 mg/mL working concentration. Both solutions are filtered through a 0.45 μm polytetrafluoroethylene (PTFE) filter before injection in the HPLC system.

3. RESULTS AND DISCUSSIONS

Currently, the internationally recognized pharmacopoeias use a gradient method for content determination of Meloxicam in injections, which can be time-consuming and not as easy as the isocratic elution method to perform. To develop a simpler and faster isocratic method for content determination of Meloxicam in injections, HPLC with UV/Vis detection was used. UV/Vis spectrophotometry was selected as the detection method based on the chemical structure of Meloxicam and its chromophores, with detection wavelength set at 360 nm, corresponding to the maximal absorbance of Meloxicam. A LiChrospher 100 RP-18 (5 μm) column from Merck was found to be the most appropriate column for the analysis. The mobile phase was optimized to achieve good separation of Meloxicam from other components in the sample. Different mobile phase components, including Methanol and Sodium hydroxide, were tested, but Acetonitrile and ultrapure water adapted with glacial acetic acid on pH of 3.1 with ratio of 60:40 was found to be the most appropriate. This ratio was chosen because the analyzed preparation is mono-component and it is advantageous for the peak to appear earlier, without any significant delay. When selecting the pH of the mobile phase, the pKa values of Meloxicam were considered. Meloxicam has two pKa values, 1.2 and 4.2. At a pH of 3.1, Meloxicam is mostly in its ionized form, which increases its solubility in the mobile phase and improves its retention time on the column. The pH was adjusted with glacial acetic acid, which is a weak acid and does not interfere with the UV detection of Meloxicam. Meloxicam has low solubility in water, but is more soluble in some organic solvents, such as Methanol. However, it was found that only a small amount of Meloxicam could be completely dissolved in Methanol. The developed isocratic method was validated and found to be quantitative, using external standard, and intended for the usage of mono-component preparations.

To determine the accuracy of the analytical method, known quantities of the standard solution were added to the sample solution to create three concentration levels (130%, 150%, and 180% of the working concentration). Three consecutive injections were made at each concentration level, and the recovery was calculated for each injection. The average recovery values for the three injections at each concentration level were found to be within the acceptance criterion, and each individual recovery value was also within the acceptance criterion. These results suggest that the analytical method is accurate in determining the content of Meloxicam in injections.

Table 1. Obtained results from accuracy

130% Spiked sample		**150% Spiked sample**		**180% Spiked sample**	
Injection	*Recovery (%)*	*Injection*	*Recovery (%)*	*Injection*	*Recovery (%)*
1	**101.12**	1	**100.60**	1	**101.12**
2	**101.29**	2	**100.75**	2	**101.97**
3	**101.37**	3	**101.37**	3	**101.76**
Average	**101.26**	Average	**100.91**	Average	**101.62**
RSD (%)	**0.13**	RSD (%)	**0.40**	RSD (%)	**0.43**

To evaluate repeatability, a standard and a sample solution, both at the working concentration, were prepared. Three consecutive injections were performed for the standard solution, and six consecutive injections were performed for the sample solution. The average value of the area under the peaks of the three consecutive injections of the standard solution was used to determine the content of the analyte in the six consecutive injections of the sample solution. The obtained results were used to calculate the RSD (1.13%), which fell within the acceptance criterion, indicating that the method is precise when performed under the same conditions, for a short period, on the same sample.

As mentioned before, the intermediate precision of the analytical method was evaluated within one day in the same laboratory by two analysts. Each analyst prepared two standard solutions and six sample solutions at the working concentration. Three consecutive injections were carried out for the standard solutions, and two consecutive injections were carried out for each sample solution. The average value of the peak area from the consecutive injections of the standard solutions and the sample solutions was calculated and used for the determination of the analyte content in each of the sample solutions. The analyte content calculation was based on the peak areas obtained from the two standard solutions individually, and the average value was considered while interpreting the results. The RSD was calculated individually for each analyst, and the variance was calculated separately for each analyst. Furthermore, the combined RSD was calculated along with the critical value at α level of significance, for 1 degree of freedom in the numerator and 22 degrees of freedom in the denominator. The RSD values, obtained individually from the results of the two analysts, and the combined RSD value (1.41%) were within the acceptance criteria. The ratio of variances between the first and second analysts (2.31) was less than the critical value (4.30), which indicates that there is no significant difference in precision between the analysts at the chosen significance level, suggesting that the method is precise when performed by two analysts, in the same laboratory, within one day.

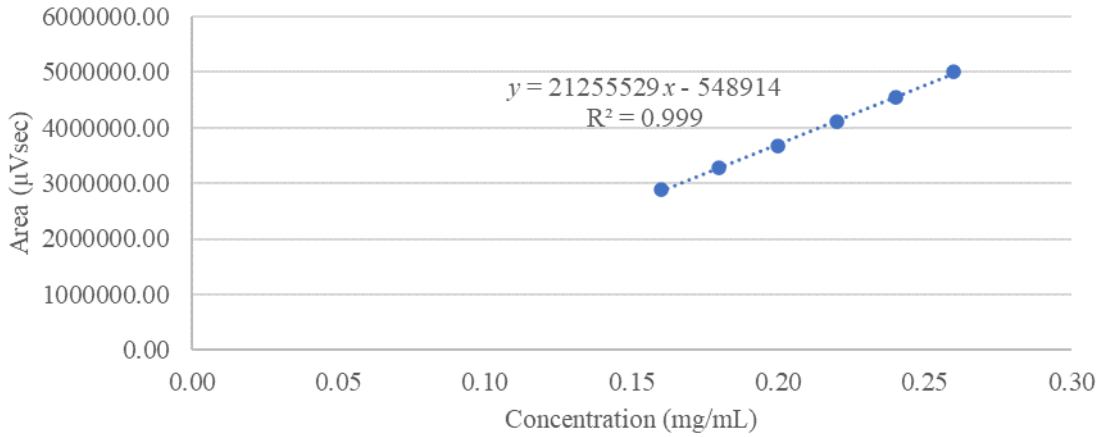
Table 2. Obtained results from intermediate precision

Results obtained from the first analyst				**Results obtained from the second analyst**			
Sample	*Content (%) according to standard solutions*		*Average content (%)*	*Sample*	*Content (%) according to standard solutions*		*Average content (%)*
1	99.56	99.20	**99.38**	1	98.33	100.06	**99.19**
2	100.60	100.23	**100.42**	2	101.04	102.81	**101.92**
3	103.09	102.72	**102.90**	3	100.85	102.63	**101.74**
4	102.44	102.07	**102.25**	4	100.38	102.15	**101.26**
5	103.18	102.80	**102.99**	5	98.86	100.60	**99.73**
6	99.40	99.04	**99.22**	6	100.60	102.37	**101.49**
Average			**101.19**	Average			**100.89**
RSD (%)			**1.72**	RSD (%)			**1.13**
Variance			**3.01**	Variance			**1.30**

To verify the specificity of the analytical method, a comparison was made between the chromatograms derived from the sample solution and the placebo solution. The sample solution was prepared by dissolving the active substance in Methanol, whereas the placebo solution was prepared by dissolving the excipients in the same solvent. Upon examination of the chromatograms, it was determined that no interferences were observed within the elution zone of the active substance in the presence of the excipients. This confirms the method's ability to accurately identify and quantify the active substance, without interference from other components.

To determine the linearity and range of the analytical method, a solution was prepared following the prescribed analytical procedure at a working concentration of 0.2 mg/mL. Additional concentrations were prepared by diluting a common stock solution, in direct correlation with the working concentration of the standard and according to the required minimum range. Ultimately, six standard solutions with varying concentrations were prepared, and three injections of each respective solution were made at each concentration level, with the average value calculated from these injections. R^2 was determined to be within the acceptance criterion, indicating a linear relationship between the independent and dependent variables. This confirms the method's ability to accurately quantify the analyte within the specified range of concentrations.

Figure 1. Graphical representation of the regression line using the least squared method



As previously mentioned, the robustness of the analytical method was assessed by monitoring three types of variations. The first type involved adjusting the ratio of the mobile phase components, with two scenarios considered. The first scenario employed a ratio of 55:45 for Acetonitrile and ultrapure water, while the second scenario used a ratio of 65:35. The second type of variation involved modifying the flow rate, which was tested at two levels: 1.3 mL/min and 1.7 mL/min. The final type of variation involved altering the pH of the ultrapure water in the mobile phase. Two scenarios were tested, with the first scenario having a pH of 2.9 and the second scenario having a pH of 3.3. The evaluation of robustness was carried out by preparing standard and sample solutions and injecting them three times for each variation. The acceptance criteria were met for each variation, indicating that the analytical method is robust.

Table 3. Obtained results from robustness

Variation	Injection	K'	A_S	N	Variation	Injection	K'	A_S	N
55:45 *(Std. sol. injections)*	1	2.493	1.388	6883.365	***65:35*** *(Std. sol. injections)*	1	3.222	1.304	5644.213
	2	2.501	1.365	6923.378		2	3.214	1.318	5525.244
	3	2.509	1.368	6906.825		3	3.187	1.338	5713.004
	RSD (%)	**0.308**	**0.900**	**0.291**		RSD (%)	**0.572**	**1.270**	**1.688**
55:45 *(Sam. sol. injections)*	1	2.519	1.339	6904.095	***65:35*** *(Sam. sol. injections)*	1	3.201	1.318	5534.913
	2	2.529	1.335	6898.638		2	3.198	1.325	5483.910
	3	2.535	1.324	6970.968		3	3.194	1.345	5433.951
	RSD (%)	**0.324**	**0.600**	**0.582**		RSD (%)	**0.112**	**1.024**	**0.920**
1.3 mL/min *(Std. sol. injections)*	1	2.371	1.313	7075.546	***1.7 mL/min*** *(Std. sol. injections)*	1	2.298	1.287	5486.364
	2	2.368	1.313	6998.395		2	2.295	1.285	5562.234
	3	2.368	1.320	6986.685		3	2.294	1.286	5576.572
	RSD (%)	**0.069**	**0.305**	**0.688**		RSD (%)	**0.100**	**0.093**	**0.875**
1.3 mL/min *(Sam. sol. injections)*	1	2.364	1.323	7039.693	***1.7 mL/min*** *(Sam. sol. injections)*	1	2.287	1.294	5448.465
	2	2.361	1.327	7021.139		2	2.285	1.300	5372.193
	3	2.353	1.340	6994.513		3	2.282	1.286	5442.800
	RSD (%)	**0.242**	**0.690**	**0.324**		RSD (%)	**0.115**	**0.563**	**0.784**
pH=2.9 *(Std. sol. injections)*	1	2.142	1.182	6292.174	***pH=3.3*** *(Std. sol. injections)*	1	2.469	1.536	5964.647
	2	2.142	1.193	6308.980		2	2.464	1.549	6058.946
	3	2.141	1.194	6278.083		3	2.461	1.548	6096.781
	RSD (%)	**0.027**	**0.557**	**0.246**		RSD (%)	**0.158**	**0.467**	**1.127**
pH=2.9 *(Sam. sol. injections)*	1	2.141	1.189	6279.238	***pH=3.3*** *(Sam. sol. injections)*	1	2.456	1.549	5928.745
	2	2.143	1.190	6301.349		2	2.453	1.562	5955.722
	3	2.143	1.194	6293.832		3	2.452	1.561	5981.052
	RSD (%)	**0.078**	**0.210**	**0.179**		RSD (%)	**0.084**	**0.454**	**0.439**

5. CONCLUSION

The method presented in this study for the content determination of Meloxicam in injections is fast, simple, cost-effective, accurate, precise, specific, linear in the given range, and robust. This method offers significant advantages in terms of its ease of use and lack of need for special preparation or training, making it valuable contribution to the field of pharmaceutical analysis. Furthermore, its potential for analyzing related drugs with similar chemical properties provides a promising solution for improving drug quality control.

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