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IMPLEMENTATION OF GREEN ANALYTICAL PRINCIPLES TO DEVELOP AND VALIDATE THE HPLC METHOD FOR THE ANTICANCER DRUG IN PHARMACEUTICAL FORMULATIONS.

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Abstract
Lenalidomide is a drug that has immune-modulating, anti-angiogenic, and anti-inflammatory properties. In this study, we developed green HPLC and spectrophotometric methods to determine the concentration of lenalidomide in pure and pharmaceutical formulations. In the HPLC method, 10 mM potassium dihydrogen phosphate solution (pH: 2.0) and ethanol (50:50, V/V) were used as mobile phases, isocratic elution was applied at a flow rate of 1.0 mL min^{-1} and detection was made at 304 nm. In the spectrophotometric method, the spectral patterns of standard solutions in different solvents were comprehensively examined, the best spectra were obtained with ultrapure water, and a wavelength of 304 nm was selected for detection. Both methods have been validated according to ICH guidelines for various parameters. Correlation coefficients greater than 0.999 were determined for both methods in the concentration range of 5–30 µg mL^{-1}. The developed methods were applied to commercial formulations, and comparisons of the results were made using the Student (t) test for means and the Fischer (F) test for standard deviations. No statistically significant difference was observed between the methods. The greenness evaluation of these methods was carried out using AGREE software. The developed methods are proposed as excellent environmental and operator-friendly alternatives for the quantification of Lenalidomide in pharmaceutical formulations.

Introduction
In today's conditions that directly affect the environment, it is vital to develop more environmentally friendly analytical methodologies. Therefore, the use of environmentally friendly chemicals is becoming more popular in all analytical techniques, including liquid chromatographic methods. When proposing an analytical method for the determination of a particular analyte, it is necessary to take into account two main characteristics. The first of these is the metrological value of the results of verification parameters. The second is that the method should be green [1–3]. Unfortunately, green analytical methods are still insufficient for pharmaceutical analysis. When developing environmentally friendly analytical techniques, guidelines that offer good ideas about green chemicals should be taken into account [4, 5].

Simple, rapid, and cost-effective analytical methods are preferred in pharmaceutical analysis. Spectrophotometric techniques are one of the most widely used techniques and continue to be popular. Spectrophotometric techniques are more convenient, more economical, and simpler to use than chromatographic techniques [6–12]. Another widely used technique in all areas of quality control analysis of pharmaceutical formulations is high-performance liquid chromatography (HPLC). This technique is more sensitive and accurate than spectrophotometric techniques. For drug analysis, HPLC methods based on reverse phase mode, which generally use hydrophobic stationary phase, polar mobile phase, and UV detector mode, have been developed in quality control laboratories. Therefore, the compatibility of the mobile phase with the detector is a parameter often considered when developing a pharmaceutical analysis technique [13].

HPLC analyses generally use a polar mobile phase and a hydrophobic stationary phase. Typical mobile phases consist of a combination of water (with additions for pH and ion balance) and organic solvents (such as acetonitrile or methanol). Acetonitrile and methanol have excellent chromatographic properties, such as complete miscibility with water, low viscosity of their aqueous solutions, low UV cutoff wavelength, low chemical reactivity with most sample types, devices, and columns, and high purity availability. These organic solvents are frequently used and preferred in HPLC analyses. However, they have some drawbacks in terms of operator health and ecological impact. Acetonitrile is a toxic, volatile, and flammable chemical. Methanol is also toxic and its waste is very difficult to dispose of. It is therefore classified as a hazardous solvent [14, 15]. Unfortunately, it is impossible to ignore the amount of waste generated during HPLC analysis. A conventional HPLC instrument generates about 1–1.5 L of waste per day, which corresponds to about 500 L of waste per year [15].

This amount is extremely modest compared to the waste generated by large factories. However, hundreds of liquid chromatographs are used in the quality control and R&D laboratories of large pharmaceutical factories. As a result, tons of toxic waste are generated every day. In addition, due to technological advances, the use of HPLC is becoming more widespread and the amount of waste is increasing at the same time. Disposal of these wastes, which contain high amounts of acetonitrile and methanol, increases the burden of laboratories and brings high costs [14–17].

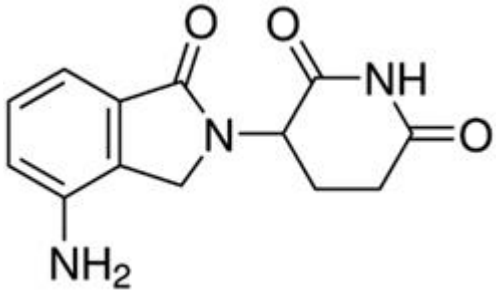
Green analytical chemists are now particularly interested in the “greening” of HPLC and spectrophotometric techniques to replace polluting analytical procedures with cleaner ones. They are working to eliminate the use of hazardous chemicals and develop environmentally and operator-friendly methods without compromising analytical method performance [18].

One of the most serious environmental challenges facing analytical chemistry is the use of solvents. Solvents should be chosen with environmental awareness [18]. During the solvent selection phase, the solubility of the drug, simplicity of sample preparation, and sensitivity of the procedure are taken into account. Generally, the most polluting step of an analytical method is the sample preparation step. Therefore, using non-polluting solvents at this stage would be an ecological approach [20]. Solvent selection recommendations of some pharmaceutical companies provide some clues for solvent optimization procedures [19].

All of these solvent selection standards are based on environmental, health, and safety concerns. Water, alcohol, and some esters are classified as "recommended" in these standards. Some substances, such as hydrocarbons and chlorinated solvents, are categorized as hazardous or non-hazardous [20].

Lenalidomide (LDM) is an immunomodulatory medication that possesses antineoplastic, antiangiogenic, and anti-inflammatory properties . It is used in the form of acetate salt because it has better bioavailability. Its chemical properties are presented in Table 1.

Table 1. The chemical properties of LDM

Properties	Value
Name	3-(4-amino-1,3-dihydro-1-oxo-2H-isoindol-2-yl)-2,6-piperidinedione
Formula	$C_{13}H_{13}N_3O_3$
Structure	
Molecular weight	259.26
Melting point	197–200 °C
Log P	−0.71
pKa (Strongest acidic)	11.61
pKa (Strongest basic)	2.31
Solubility	Soluble in aqueous solvents and organic solvent/water combinations.

In the literature, LDM was detected by spectrophotometry in bulk and dosage forms, by fluorimetry in bulk and capsules, by capillary electrophoresis in pharmaceutical preparations by high-performance liquid chromatography in biological fluids and pharmaceutical formulations, by liquid chromatography coupled with mass spectrometry in biological fluids, by liquid chromatography coupled with mass spectrometry in rabbit and human plasma. Most of these methods are highly complex and require toxic organic solvents and specialized chemicals. The sample preparation steps of these techniques are complex and require long run times and gradient elution.

Therefore, this study aimed to develop and validate an environmentally and analyst-friendly liquid chromatography method using ethanol as a mobile phase organic solvent and a spectrophotometric method using pure water as the solvent for LDM quantification in pharmaceutical products with a simple extraction procedure. This study describes a new chromatographic method using the less

hazardous solvent ethanol. Furthermore, this study demonstrates how It is simple to replace solvents with “less hazardous” and “greener” ones with satisfactory performance.

Experimental

2.1 Instruments and software

On an HPLC system (Agilent 1260, USA) outfitted with a UV–Vis detector, quaternary pump, vacuum degasser, column oven, and Chemstation software, chromatographic studies were carried out. Using 1.0 cm quartz cuvettes and UV-Probe software, spectrophotometric measurements were carried out on a dual beam path spectrophotometer (Shimadzu UV-1800, Japan). An Agilent Extend C_{18} (250 × 4.6 mm, 5 μm) column was used for chromatographic separation. pH measurements were made with a pH meter (Mettler-Toledo, Switzerland) equipped with a glass electrode. Ultrapure water was produced with a water purification system (Millipore Milli-Q, USA).

2.2 Materials and reagents

All solvents were gradient purity for liquid chromatography. Lenalidomide (LDM) (≥98.0%), acetonitrile (≥99.9%), methanol (≥99.0%), ethanol (≥99%), and analytical grade potassium dihydrogen phosphate (≥99.0%) were purchased from Sigma-Aldrich Chemie GmbH (Istanbul, Turkey). LDM capsules (Revlimid, 10 mg) used in this study were purchased from a local pharmacy (Afyonkarahisar, Turkey).

Each capsule contains 10 mg of lenalidomide. In addition, each capsule contains lactose anhydrous, microcrystalline cellulose, croscarmellose sodium, magnesium stearate, gelatin, shellac, propylene glycol, potassium hydroxide, titanium dioxide (E171), black iron oxide (E172) as excipients. Ultrapure water (0.075 μS cm^{-1}) was used for the preparation of all solutions and the mobile phase. Before analysis, the mobile phase was filtered through a 0.45 m membrane filter using a vacuum pump and sonicated.

2.3 Solutions

Stock standard solution (500 μg mL^{-1}): 25 mg of the reference LDM standard was accurately weighed, transferred to a 50 mL volumetric flask, topped up with 20 mL of ultrapure water, sonicated for 10 min to ensure dissolution, and the volume was topped up to 50 mL with ultrapure water.

Standard solutions (5, 10, 15, 20, 25, and 30 μg mL^{-1}): Different volumes of the stock solution were subjected to serial dilutions with ultra-pure water to obtain six standard solutions in the concentration range of 5–30 μg mL^{-1}.

Sample solution (20 μg mL^{-1}): The contents of 10 capsules (Revlimid, 10 mg) were emptied and weighed accurately, and the mass of an average capsule was recorded, crushed in a dry and clean mortar, ground to a fine powder, and mixed. The capsule powder equivalent to 50 mg of LDM was precisely weighed and transferred to a 100 mL volumetric flask. Approximately 40 mL of ultrapure water was added and shaken on a rotary shaker for 30 min to ensure complete dissolution. The volume was topped up with ultrapure water. The mixture was sonicated for 10 min and then filtered through a 0.45 mm membrane filter. This solution was called the stock sample solution. A sample solution was prepared at a concentration of 20 μg mL^{-1} by diluting the stock sample solution with ultrapure water.

2.4 Determination of λ_{max}

To determine the λ_{max} value, standard solutions in the concentration range of (5–30 μg mL^{-1}, n = 6) were scanned against ultrapure water in the wavelength range of 200–800 nm in a spectrophotometer device.

2.5 Development of methods

The chromatographic conditions were optimized to obtain good peak parameters such as a good peak shape, the lowest tailing factor, a short retention time, and a high theoretical plate number. Initially, mobile phases consisting of various buffer systems were investigated, but the required system compatibility characteristics could not be achieved. Different types (X-Terra C_{18} (250 × 4.60 mm × 5 µm), Extend C_{18} (250 mm × 4.6 mm, 5 µm), Synergy Hydro C_{18} (250 mm × 4.6 mm × 4 µm) and Luna C_{18} (250 mm × 4.6 mm × 5 µm)) and different lengths of columns (X-Terra C_{18} (150 × 4.60 mm × 5 µm), Extend C_{18} (150 mm × 4.6 mm, 5 µm), Synergy Hydro C_{18} (150 mm × 4.6 mm × 4 µm) and Luna C_{18} (150 mm × 4.6 mm × 5 µm)) were tested but showed poor system compatibility parameters. Good peak parameters were obtained using an Extend C_{18} (250 mm × 4.6 mm, 5 µm) column. Different ratios of water/methanol, water/acetonitrile, and water/ethanol mixtures were tested as mobile phases. Initially, acetonitrile and ultrapure water (20/80, V/V) were used as mobile phase, which resulted in a very long analysis time. To shorten the analysis time, the water component of the mobile phase was acidified with formic acid (pH:2.0).

Under these conditions, the sample solution was injected to detect both impurities that may interfere with the analyte peak and the presence of drug matrix components that may remain longer on the column under the specified conditions. Furthermore, the sample solutions were injected sequentially into the system with an analysis time of 10 min and it was observed that no impurities were carried over from one analysis to the next. Therefore, the analysis time was set to 10 min. Furthermore, the column temperature was chosen as 30 °C due to its many advantages such as high column efficiency, low column pressure favorable peak shape, and cost-effectiveness. The spectral pattern of LDM was comprehensively investigated using different solvents (ultrapure water, ethanol, methanol, and isopropyl alcohol) for spectrophotometric analysis. Ultra-pure water was used as the solvent for spectrophotometric analysis since the best spectra of LDM were obtained with ultrapure water. Since the standard solutions of LDM have maximum absorbance at 304 nm wavelength, the absorbance values of the standard and sample solutions were measured at this wavelength.

2.6 Validation of analytical methods

Analytical methods developed for the quantification of LDM have been validated for “selectivity, system suitability, linearity, precision, sensitivity, and robustness” according to the ICH Q2 (R1) guidelines .

Standard, sample, and mobile phase solutions were injected into the chromatographic system to evaluate the selectivity of the chromatographic method. The retention time (Rt) of LDM in commercial formulations and sample solution chromatograms was evaluated by comparing them with the chromatograms of the standard solution. To evaluate the selectivity of the spectrophotometric method, spectra of the standard, sample, and solvent (ultrapure water) were taken in the wavelength range of 200–400 nm in a spectrophotometer. The spectra obtained were compared and the presence of interfering bands was analyzed.

To evaluate the system suitability of the chromatographic method, LDM standard solution (25 µg mL^{-1}) was injected into the chromatographic system six times at short regular time intervals. Peak area, retention time, tailing factor, and the number of theoretical plates were recorded from the chromatograms. Relative standard deviation values were calculated for peak area and retention time. To evaluate the system suitability of the spectrophotometric method, absorbance values of LDM standard solution (20 µg mL^{-1}, n = 6) were measured. The relative standard deviation of the absorbance values was calculated.

The linearity of the chromatographic method was determined by injecting six standard solutions in the concentration range of 5–30 µg mL^{-1} into the HPLC system for three replicates on three different

days. The chromatographic responses (peak areas) obtained for each concentration were recorded. Calibration curves were plotted with concentrations on the x-axis and chromatographic responses (peak areas) on the y-axis. The linearity of the spectrophotometric method was determined by measuring the absorbance values of six standard solutions in the concentration range of 5–30 μg mL^{-1} in the spectrophotometer for three replicates on three different days. The spectrophotometric responses (absorbance values) obtained for each concentration were recorded. Calibration curves were plotted with concentrations on the x-axis and spectrophotometric responses (absorbance values) on the y-axis. The data from both analytical methods were used for regression analysis, which was done using the least squares method. The linearity of the method was measured by the absolute mean recovery, RSD, and R2 of the calibration curve.

The sensitivity of the methods was evaluated by calculating the limits of detection (LOD) and limits of quantification (LOQ). The detection and quantification limits were determined from the standard deviation of the intercept and slope of the calibration curve using the equations (LOD=3.3xσ/S) and (LOQ=10xσ/S). In these equations, σ is the standard deviation of the point where the calibration curve crosses the y-axis, while S is the slope of the calibration curve.
The accuracy of the analytical methods was assessed by determining the % recovery values of the added analyte using the "standard addition method". To a pre-analyzed LDM solution (10 μg mL^{-1}), an additional 75%,100%, and 125% LDM standard was added to obtain concentrations of 20 μg mL^{-1}. The resulting solutions were re-quantitatively analyzed using the developed methods. The amounts of LDM recovered were determined and % accuracy values were calculated (n = 6).

The analytical methods' accuracy was assessed using their intra-day and inter-day precision. For both procedures (n = 3), intra-day precision was assessed using quantitative measurement of a standard solution at a concentration of 20 g mL^{-1} on the same day. Inter-day precision was determined by quantitative analysis of a standard solution at a concentration of 20 μg mL^{-1} on three different days for both methods (n = 9). In the chromatographic method, peak areas and retention times were recorded and relative standard deviation values were calculated. In the spectrophotometric method, absorbance values were measured and relative standard deviation values were calculated.

The robustness of the chromatographic method was assessed by making small deliberate changes to the method conditions. Small modifications were made to the flow rate of the mobile phase (±0.1 mL min^{-1}), the organic modifier content in the mobile phase (±2%), the detection wavelength (±2 nm), and the effect of these modifications on the system suitability parameters were observed. To establish the system suitability parameters following each alteration, a standard solution (20 g mL^{-1}) was injected into the chromatographic system. The results were then compared to the results obtained under the original chromatographic circumstances. These effects were studied by three replicate analyses of the standard solution. Little changes were performed to the organic solvent (ethanol and isopropyl alcohol) and the detection wavelength (302 and 306 nm) in order to evaluate the spectrophotometric method's robustness. The results were compared with the results under the original spectrophotometric conditions. These effects were studied by three replicate analyses of the standard solution.

2.7 Application of analytical methods to commercial formulations

The contents of 10 capsules (Revlimid, 10 mg) were emptied and weighed accurately, and the mass of an average capsule was recorded, crushed in a dry and clean mortar, ground to a fine powder, and mixed. For quantitative analysis of commercial formulations of LDM, tablet powder containing 25 mg LDM was accurately weighed, dispersed in 50 mL of ultrapure water, and sonicated for 10 min. It was filtered to remove undissolved substances. This solution was referred to as the sample stock solution. The sample solution was prepared by taking 400 μL of the stock solution and diluting it to 10 mL with

ultrapure water. The solutions were quantitatively analyzed by chromatographic and spectrophotometric methods.

2.8 Greennes profiling of analytical techniques

A metric system (AGREE) was used to assess the greenness of analytical methods (available at: https://git.pg.edu.pl/p174235/AGREEhttps://git.pg.edu.pl/p174235/AGREE). AGREE includes 12 basic principles of greenness assessment and allows weight assignment. It has user-friendly software, is easy to implement, and provides easy-to-interpret color pictogram output showing strengths and weaknesses. The AGREE score is a weighted average of the benchmark scores. It is shown in the center of the graph and its value ranges from 0.0 (lowest score) to 1.0 (perfect score). The graph is a visual representation of the score itself, the benchmark scores, and the benchmark weights .

Results

3.1 Determination of the wavelength

Standard solutions prepared using ultrapure water were scanned in a spectrophotometer device in the 200–800 nm wavelength range. The maximum absorption wavelength of LDM was determined as 304 nm .

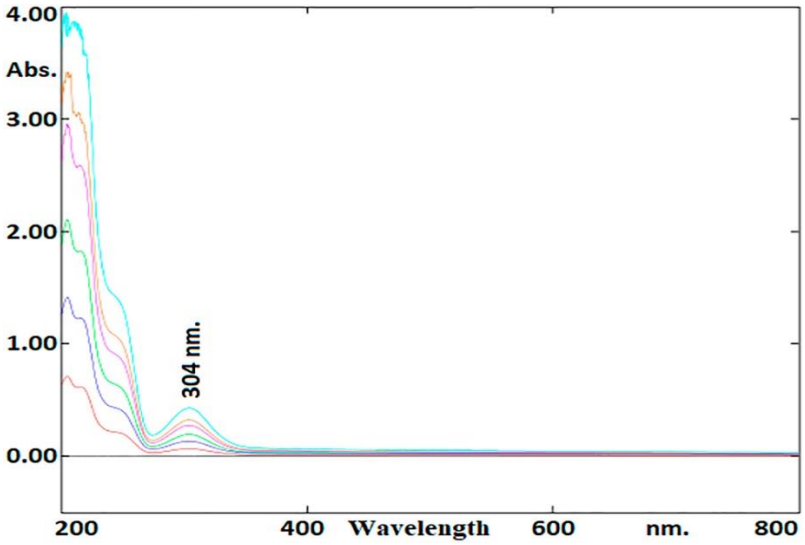


Fig. 1. The overlaid spectrum of LDM standard solutions in the 5–30 µg mL^{-1} concentration range

3.2 Development of methods

The conditions of the developed analytical methods are given below.

Extend C18 column (250 × 4.6 mm, 5 µm) at a constant temperature of 25 °C was used for separation in the chromatographic method. The mobile phase contained 10 mM potassium dihydrogen phosphate solution (pH: 2.0 with orthophosphoric acid) and ethanol (50:50, V/V).

At a flow rate of 1.0 mL min^{-1}, isocratic elution was carried out and 304 nm was chosen for detection. Spectrophotometric method; the spectral pattern of LDM was extensively analyzed. The best LDM spectra were obtained with ultrapure water and this solvent was used for spectrophotometric analysis. To determine the wavelength at which LDM standard solutions maximally absorbed UV light, the standard solutions were scanned in the 200–400 nm wavelength range. A wavelength of 304 nm was selected for detection.

3.3 Validation of analytical methods

The analytical methods developed for the quantification of LDM in oral formulations were validated for “selectivity, system suitability, linearity, precision, sensitivity, robustness, and specificity” according to ICH Q2 (R1) guidelines.

Standard, sample, and mobile phase solutions were injected into the chromatographic system to evaluate the selectivity of the chromatographic method. The interference peak(s) around the analyte peak were identified by comparing the three chromatograms. No peak interfering with the LDM retention time was observed in all chromatograms. The overlapping chromatogram of the standard, sample, and mobile phase solution is presented in Fig. 2.

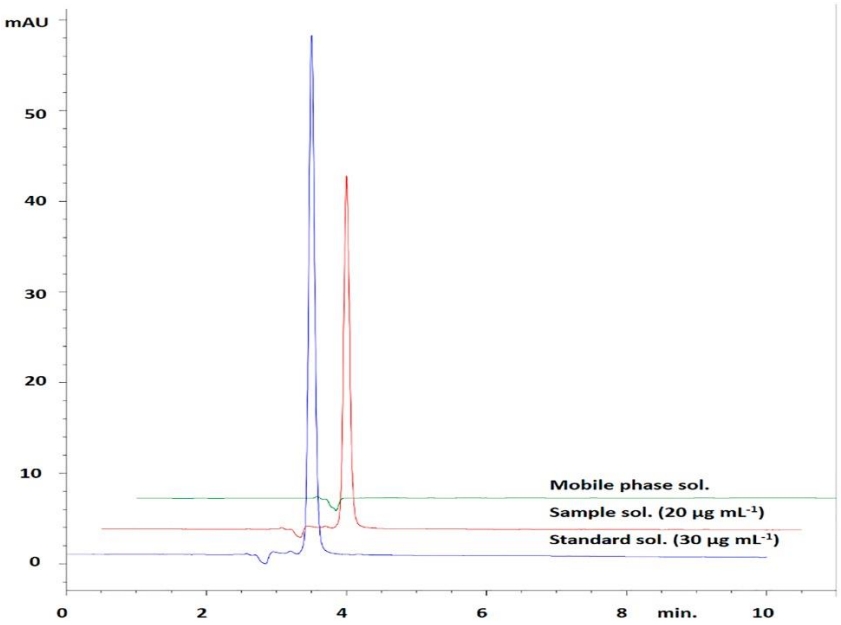


Fig. 2. Chromatogram of standard (30 µg mL^{-1}), sample (20 µg mL^{-1}), and mobile phase solution

To evaluate the selectivity of the spectrophotometric method, spectra of standard solutions (5, 10, 15, 20, 25, 30 µg mL^{-1}), sample solution, and ultrapure water were scanned in the wavelength range 200–800 nm. The spectra were compared and the spectrum of the sample solution was analyzed for spectral band(s) interfering with the analyte spectrum. No bands interfering with the LDM bands were observed in the spectrum of the sample solution. The overlapping spectra of the standard solutions produced by the spectrophotometric method and the spectrum of the sample solution are presented in Figs 1 and 3, respectively.

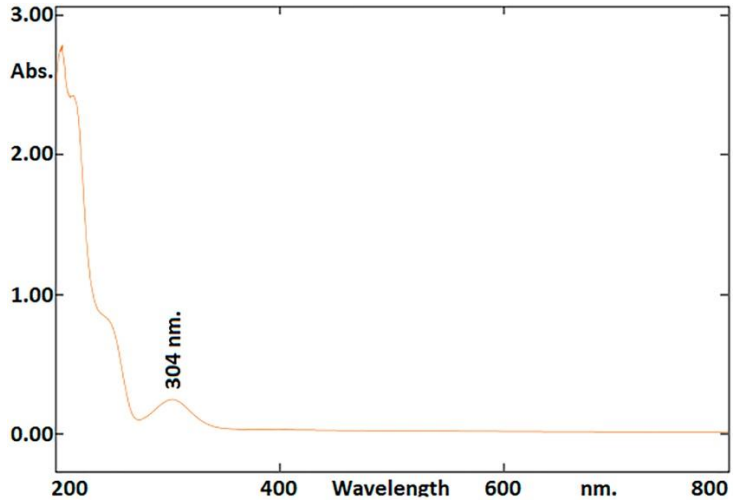


Fig. 3. Spectrum of sample solution (20 µg mL^{-1})

A standard solution (n = 6) containing 20 g mL^{-1} was injected into the system, and the primary parameters were determined, to assess the system suitability of the chromatographic method.

LDM has excellent peak symmetry and the peak areas and retention times showed consistently low variability. To determine the spectrophotometric method's system suitability, absorbance values of the standard solution ($n = 6$) at a concentration of 20 μg mL^{-1} were used to measure the results in a spectrophotometer. LDM absorbance values consistently showed low variability. The calibration curve's correlation coefficient in this research was 0.9999 for the chromatographic method and 0.9998 for the spectrophotometric method. This indicates that the methods are suitable for samples with highly complex matrices. The determined values are listed in Table 2. Therefore, the developed methods are highly suitable for LDM quantification in pharmaceuticals.

Table 2. The results of the system suitability tests

Sample	Liquid chromatography technique				UV spectrophotometry technique
	Peak area	Retention time	Peak tailing	Teoric plate count	Absorbance
1	236.75	3.498	1.082	6,154	0.268
2	238.21	3.497	1.113	6,125	0.272
3	237.01	3.496	1.093	6,118	0.269
4	237.61	3.495	1.093	6,152	0.271
5	235.84	3.498	1.089	6,150	0.267
6	238.04	3.497	1.105	6,167	0.273
Average value	237.24	3.497	1.096	6,144	0.270
S. D.	0.891	0.001	0.011	19	0.002
R. S. D.	0.386	0.033	1.027	0.306	0.876

By diluted the stock solution of the standard (500 μg mL^{-1}) with ultrapure water, standard solutions (5, 10, 15, 20, 25, and 30 μg mL^{-1}) were organized in triplicate. For the chromatographic method, the standard solutions were injected into the system. The peak areas and retention times of the analyte were recorded. Average peak areas were calculated for each concentration level. A calibration graph was constructed with the peak area values versus the concentration of the standard solution. For the spectrophotometric method, the absorbance values of the standard solutions were measured against the blank solution. The average absorbance value for each concentration level was calculated. A calibration graph was plotted from absorbance data against standard solution concentration. Regression analysis was used to assess the linearity of the analytical data. The regression equation,

slope, and intercept were calculated using linear regression analysis based on the least squares method. The results of the linearity studies are presented in Table 3. In the concentration range of 5–30 μg mL^{-1}, the calibration curve demonstrated a good linear relationship. In this study, the correlation coefficient of the calibration curve was 0.9999 for the chromatographic method and 0.9998 for the spectrophotometric method.

Table 3. Regression data of analytical techniques

Parameter	Liquid chromatography technique	UV spectrophotometry technique
Linearity concentration range (μg mL^{-1})	5–30	5–30
Regression equation (y = mx + n)		
Slope (m)	11.53	0.014
Intercept (n)	6.1773	−0.0101
Correlation coefficient (r^2)	0.9999	0.9998
LOD (μg mL^{-1})	0.30	0.70
LOQ (μg mL^{-1})	1.00	2.10
Recovery % [n = 6]	99.48–100.15	98.71–100.79

The sensitivity of the methods was evaluated by calculating the limits of detection (LOD) and limits of quantification (LOQ). Detection and quantification limits were determined as 0.10 and 0.30 for the HPLC method and 0.70 and 2.10 for the spectrophotometric method, respectively. The chromatogram of the LDM standard solution at LOQ concentration is presented in Fig. 4 and its spectrum is presented in Fig. 5.

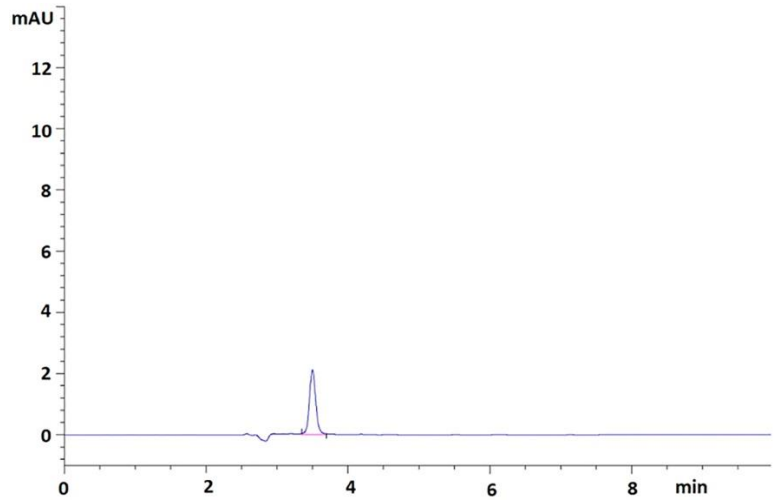


Fig. 4. Chromatogram of standard solution (LOQ concentration)

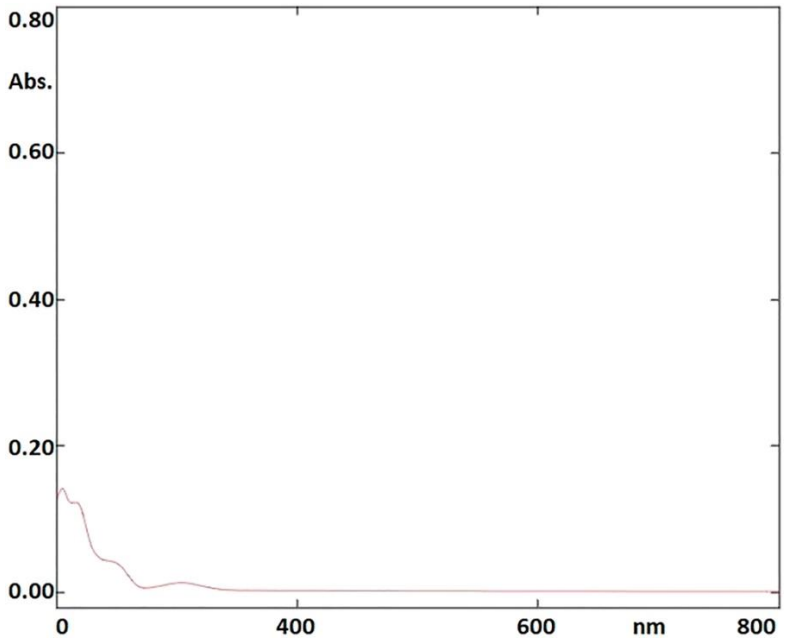


Fig. 5. Spectrum of sample solution (LOQ concentration)

Intraday precision was determined by recording the areas and retention times of LDM peaks obtained from three injections of the reference solution with a 20 g mL^{-1} concentration on the same day in the chromatographic method (n = 3). In the spectrophotometric method, the absorbance values of the standard solution with a concentration of 20 µg mL^{-1} on the same day were recorded (n = 3). Inter-day precision was measured by recording the areas and retention times of LDM peaks obtained from three injections of 20 µg mL^{-1} standard solution on three days in a row (n = 9) in the chromatographic technique. In the spectrophotometric method, absorbance values of 20 µg mL^{-1} standard solution were recorded for three consecutive days (n = 9). Peak areas, retention times, and the absorbance values' relative standard deviation were computed. Intra-day precision and inter-day precision results are given in Table 4. It was observed that the relative standard deviation values of peak areas, retention times, and absorbances of the analytical methods were below 1.00%. Our data show that the methods fulfill the validation requirements.

Table 4. Precision results of analytical techniques

Precision	Sample No	Liquid chromatography technique			UV spectrophotometry technique	
		Retention time min.	Peak Area	Assay %	Absorbance	Assay %
Intra-day	1	3.494	236.75	99.76	0.268	99.38
	2	3.495	238.21	100.37	0.272	100.87
	3	3.498	237.01	99.87	0.269	99.75
	Mean	3.496	237.32	100.00	0.270	100.00
	S.D.	0.002	0.779	0.328	0.002	0.772
	R.S.D.	0.060	0.328	0.328	0.772	0.772
Inter-day	1	3.494	236.75	99.81	0.268	99.30
	2	3.495	238.61	100.59	0.272	100.78
	3	3.498	237.01	99.92	0.269	99.67
	4	3.500	236.32	99.63	0.271	100.41
	5	3.501	237.15	99.98	0.267	98.93
	6	3.496	236.33	99.63	0.273	101.15
	7	3.493	237.94	100.31	0.272	100.78
	8	3.495	238.37	100.49	0.268	99.30
	9	3.494	236.35	99.64	0.269	99.67
	Mean	3.496	237.20	100.00	0.270	100.00
	S.D.	0.003	0.894	0.377	0.002	0.796

Precision	Sample No	Liquid chromatography technique			UV spectrophotometry technique	
		Retention time min.	Peak Area	Assay %	Absorbance	Assay %
	R.S.D.	0.081	0.377	0.377	0.796	0.796

Three different concentrations of the LDM standard were added to the sample solution to test the accuracy of the analytical techniques. The standard was added to the sample solution (20 μg mL^{-1}) at the proportion of 75, 100, and 125% of the LDM content. The resulting solutions were analyzed by analytical methods. The % recovery values of the amount of standard added were calculated. Each concentration was tested three times. The recovery percentages ranged between 99.63% and 100.00% for the chromatographic method and between 99.45 and 99.85% for the spectrophotometric method. The maximum relative standard deviation values were 0.296 for the chromatographic method and 0.490 for the spectrophotometric method. Table 5 displays the findings of the recovery investigations.

Table 5. Accuracy results of analytical techniques

Technique	Standard addition level %	Standard addition amount μg mL^{-1}	Average recovery %	S. D.	R. S. D.
Liquid chromatography technique	75	15	99.63	0.295	0.296
	100	20	99.86	0.203	0.203
	125	25	100.00	0.145	0.145
UV spectrophotometry technique	75	15	99.45	0.487	0.490
	100	20	99.71	0.284	0.285
	125	25	99.85	0.256	0.256

To assess the robustness of the methods, small deviations from the optimal values of both analytical methods were made and these changes were observed to have an impact on the system suitability parameters. To determine the system suitability parameters in the chromatographic procedure, 20 g mL-1 of a standard solution was injected into the chromatographic system after each changes. The results were then compared to the results obtained under the original chromatographic circumstances. A reference solution (20 μg mL^{-1}) was injected into the HPLC system for the chromatographic method. In the spectrophotometric method, the effect of different solvents and detection wavelengths was studied and compared with the results under the original spectrophotometric conditions. These effects were studied by three replicate analyses of the standard solution. Small deviations from the optimum values for the method parameters did not have a significant effect on the results. According to the results obtained, the largest relative standard deviation value was calculated to be 0.44 (Table 6).

Table 6. The results of robustness tests for analytical techniques (*n* = 3)

Method	System conditions	Values	Average recovery %	R.S.D. %
Liquid chromatography technique	Normal conditions		100.02	0.33
	The high flow rate of the mobile phase	1.10 mL min^{-1}	99.70	0.40
	The low flow rate of the mobile phase	0.90 mL min^{-1}	99.55	0.44
	High detection wavelength	306 nm.	99.73	0.26
	Low detection wavelength	302 nm.	99.78	0.36
	The high ethanol content (mobile phase)	52%	99.75	0.29
	The low ethanol content (mobile phase)	48%	99.62	0.32
UV spectrophotometry technique	Normal conditions		99.93	0.39
	High detection wavelength	306 nm.	99.56	0.37

Method	System conditions	Values	Average recovery %	R.S.D. %
	Low detection wavelength	302 nm.	99.37	0.48
	Solvent	Ethanol	99.48	0.43
	Solvent	Isopropyl alcohol	99.20	0.41

3.4 Application of analytical methods to pharmaceutical formulations

6 capsules (Revlimid, 10 mg) were quantitatively analyzed by the developed analytical methods. Table 7 shows the findings of both analytical methods, relative standard deviation, standard deviation and the mean, derived over six replicates. The Student (t) test and the Fischer (F) test were used to compare the outcomes of the two methods in terms of means and standard deviations, respectively. When the results in the table are analyzed, in terms of accuracy and precision, it can be shown that there is no noticeable distinction between the two analytical approaches. The *t* and F values obtained as a result of 6 trials were below the values indicated in the relevant tables.

Table 7. Statistical evaluation of analysis results of LDM capsules (Revlimid, 10 mg)

Sample	Liquid chromatography technique		UV spectrophotometry technique	
	mg/tablet	%	mg/tablet	%
1	9.77	98.29	10.26	102.53
2	9.62	96.78	9.93	99.23
3	10.02	100.80	10.13	101.23
4	9.99	100.50	9.98	99.73
5	9.93	99.90	10.00	99.93
6	10.31	103.72	9.74	97.34
Average	9.94	100.00	10.01	100.00
S.D.	0.24	2.37	0.18	1.77

Sample	Liquid chromatography technique		UV spectrophotometry technique	
	mg/tablet	%	mg/tablet	%
R.S.D	2.37	2.37	1.77	1.77
t_{value}/t_{table}	0.1218/2.7764			
F_{value}/F_{table}	3.0269/6.3882			

3.5 Greennes profiling of analytical techniques

The pictograms of the greenness assessment of the analytical techniques are given in Fig. 5. The greenness score of the chromatographic technique is 0.75, while the spectrophotometric technique has a greenness score of 0.76. The scores relating to the green analytical chemistry standards 1, 7, and 8 in the AGREE pictogram of the chromatography technique were rather low, whereas the scores relating to the principles 4, 6, 9, 10, and 11 were extremely good (Fig. 6A). In the AGREE pictogram of the spectrophotometric technique, the scores for GAC principles 1 and 5 are quite low, while the performance for principles 3, 4, 6, 10, 11, and 12 is excellent (Fig. 6C). It can be said that both analytical methods are green, however, the chromatographic technique is more environmentally conscious than the spectrophotometric approach. The corresponding color scale for the reference is presented in Fig. 6B.

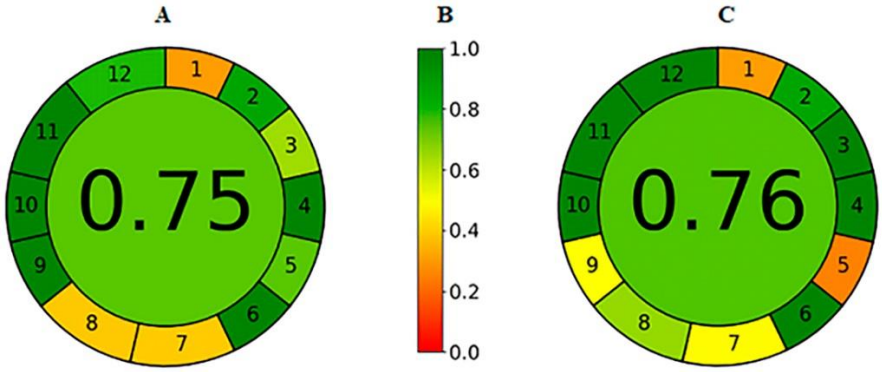


Fig. 6. A: AGREE pictogram of the chromatographic method, B: The color scale for reference, C: AGREE pictogram of the spectrophotometric method

Discussion

There is no green analytical method for quantification of LDM for pharmaceutical products in previous studies.

In this research, environmentally friendly chromatographic and spectrophotometric methods were developed for the determination of LDM in pharmaceutical products and operatör-friendly chemicals that meet all the requirements of the validation process without compromising the quality of chromatographic and spectrophotometric performance. Analytical methods reported for the determination of LDM in pharmaceutical products are compared. The environmental friendliness of the analytical methods was evaluated from sample preparation to detection. In other reported methods, toxic chemicals were used during sample preparation. The use of hazardous substances was avoided in the sample preparation phase of the developed analytical methods.

The use of environmentally and operator-friendly chemicals in sample preparation and mobile phase provided an alternative perspective. In all previous studies, methanol or acetonitrile was used as the mobile phase of the HPLC analysis. The retention periods of LDM, as evaluated by the green technique, are longer in various HPLC procedures described in the literature. Too much waste is produced as a result. However, as compared to the suggested procedure, the described analytical techniques are not environmentally friendly. The developed technique was found to be more environmentally friendly in terms of solvent and reagent risks after using a green evaluation tool to evaluate and compare its more environmentally friendly nature. The findings showed that the green quantification of LDM in pharmaceutical products was performed without loss of chromatographic quality due to reduced harmful effects. Such green analysis methods will encourage analysts interested in developing more environmentally friendly analysis methods in their laboratories.

Conclusions

Developing environmentally friendly methods to prevent environmental pollution, and reduce energy consumption and waste generation has become critical for the future of humanity. With this in mind, environmentally and operator-friendly spectrophotometric and chromatographic techniques can be developed for LDM quantification in pharmaceutical products without compromising method performance quality. No toxic solvents were used at any stage of these techniques, including sample preparation. The developed analytical methods met all the requirements of the validation process according to ICH guidelines and were observed to be linear, accurate, sensitive, robust, and responsive.

Safe and economical organic solvents such as ultrapure water and ethanol were used in both the sample preparation and detection stages of the developed methods. Furthermore, the greenness profile score of the developed methods is significantly better than other published methods. We believe that this study will be an example of such studies. Therefore, the proposed method can be considered as an advantageous and innovative method in the application of green analytical chemistry as an ecologically safe and accurate alternative for use in routine quality control analysis.

References

1. Armenta, S.; Garrigues, S.; De la Guardia, M. Green analytical chemistry. TRAC-Trend Anal. Chem. 2008, 27, 497–511. https:// doi.org/10.1016/j.trac.2008.05.003.
2. Koel, M.; Kaljurand, M. Application of the principles of green chemistry in analytical chemistry. Pure Appl. Chem. 2006, 78, 1993–2002. https://doi.org/10.1351/pac200678111993.
3. Tobiszewski, M.; Mechlinska, A.; Namiesnik, J. Green analytical chemistry – theory and practice. Chem. Soc. Rev. 2010, 39, 2869–78. https://doi.org/10.1039/B926439F.
4. Gałuszka, A.; Migaszewski, Z.; Namiesnik, J. The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices. TRAC-Trend, Anal. Chem. 2013, 50, 78–84. https://doi.org/10.1016/j.trac.2013.04.010.
5. Płotka, J.; Tobiszewski, M.; Sulej, A. M.; Kupska, M.; Gorecki, T.; Namiesnik, J. Green chromatography. J. Chromatogr. A. 2013, 1307, 1–20. https://doi.org/10.1016/j.chroma.2013.07.099.
6. Sangoi, M. S.; Todeschini, V.; Steppe, M. Second-order derivative UV spectrophotometric method for the determination of fesoterodine and comparison with LC, CE, and LC-MS/MS in commercial extended-release tablets. Acta Chim. Slov 2012, 59, 136–43.
7. Ashour, S.; Bayram, R. Selective and validated kinetic spectrophotometric method for the determination of irbesartan in pure and pharmaceutical formulations. Ann. Pharm. Fr 2019, 77(2), 101–11.
8. Corte, A. C.; Sfair, L. L. Development and validation of analytical methodology for quality evaluation of tibolone in capsule pharmaceutical form through UV spectrophotometry. Drug Anal. Res. 2019, 3(2), 7–11.

9. Gomes, P.; Negretto, C. M. U.; Naisinger, Z. B.; Lorenzoni, R.; Wingert, N. R.; Raffin, R. P. Second-derivative spectrophotometry for the analysis of simvastatin in polymeric Nanocapsules. Drug Anal Res. 2019, 3(2), 12–7.
10. Oppe, T. P.; Menegola, J.; Schapoval, E. E. S. Development and validation of UV spectrophotometry and liquid chromatography methods for determination of cefpirome in raw material and pharmaceutical dosage. Drug Anal Res. 2019, 3(1), 42–50.
11. Naguib, A. I.; Abdelaleem, E. A.; Hassan, E. S. Comparative study of eco-friendly spectrophotometric methods for accurate quantification of mebendazole and quinfamide combination; Content uniformity evaluation. Spectrochim Acta A 2020, 235, 1–10.
12. Darwish, H. W.; Ali, N. A.; Naguib, I. A.; Ghobashy, M. R. E.; Al-Hossaini, A. M.; Abdelrahman, M. M. Stability indicating spectrophotometric methods for the quantitative determination of bromazepam and its degradation product. Spectrochim Acta A 2020, 238, 1–10.
13. Görög, S. The changing face of pharmaceutical analysis. TrAC Trends Anal. Chem. 2007, 26, 12–7.
14. Snyder, L. R.; Kirkland, J. J.; Dolan, J. W. Introduction to Modern Liquid Chromatography; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2009, ISBN 978-0-470-50818-3.
15. Welch, C. J.; Wu, N.; Biba, M.; Hartman, R.; Brkovic, T.; Gong, X.; Helmy, R.; Schafer, W.; Cuff, J.; Pirzada, Z. Greening analytical chromatography. TrAC Trends Anal. Chem. 2010, 29, 667–80.
16. ICH Harmonised Tripartite Guideline Impurities: Guideline for residual solvents Q3C (R5). Curr. Step 2005, 4, 509.
17. Sheldon, R. A. Fundamentals of green chemistry: efficiency in reaction design. Chem. Soc. Rev. 2012, 41, 1437–51.
18. Tobiszewski, M. Metrics for green analytical chemistry. Anal Methods 2016, 8, 2993–9.
19. Prat, D.; Hayler, J.; Wells, A. A survey of solvent selection guides. Green. Chem. 2014, 16, 4546–51.
20. Ette, E.; Zhou, S.; Weiss, D.; Palmisano, M. Population pharmacokinetics and exposure-safety of lenalidomide in patients with multiple myeloma, myelodysplastic syndromes, and mantle cell lymphoma. Blood 2013, 122, 3234. https://doi.org/10.13140/RG.2. 2.16459.08481