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Research Article

Comparation with Spectrophotometric and Liquid Chromatographic Methods of Pharmaceutical Forms of Ivermectin

Numan Taspinar

Uşak University, Faculty of Medicine, Department of Medical Pharmacology, Uşak, Türkiye

Abstract

Aim: Ivermectin is a semi-synthetic parasiticide that is used to treat parasitic disorders. Herein, In this study, LC chromatographic and UV spectrophotometric methods were developed and validated for the determination of ivermectin in different ivermectin preparations.

Materials and Methods: In the LC chromatografic method, chromatographic separation was performed using an Agilent Extend-C18 column. Acetonitrile (20/80, v/v) and ultra-pure water was used as mobile phase at a flow rate of 1.2 mL/min. Eluents were determined at a wavelength of 245 nm and the values of ivermectin solutions were detected by spectrophotometric technique at the same wavelength. Lambert–Beer plots showed linear relationships at 6 different concentrations in the range of 10 to 60 µg/mL. Both methods adhered to the protocols published by ICH guidelines Q2(R1) to validate analytical methods.

Results: The developed analytic methods were statistically validated. As a result of the analyzes performed with spectrophotometric and liquid chromatographic methods, it was determined that both methods were precise, accurate and robust with a RSD < 1% result. Recovery values were within the normal range (98-100%). Statistical comparison of both analytical methods was made and there was no statistical significance between them.

Conclusion: These developed methods have been found to be reliable, fast, accurate and simple for tablet and injectable ivermectin forms and can be used for quality control tests. HPLC and UV spectrophotometric methods have shown that they are both adequate to determine the amount of ivermectin in raw materials, tablets and injectable solutions. These methods can be applied in a short time and easily. They can be used successfully in quality control analyzes to quantify and identify ivermectin in marketed formulations.

Keywords: Ivermectin, HPLC, UV, method, validation

INTRODUCTION

Few treatment protocols exist to reduce morbidity and mortality from COVID-19. Although corticosteroids have been shown to reduce mortality in severe diseases caused by COVID-19, no precise treatment data can prevent the disease and reduce hospitalizations and deaths (1). Ivermectin is used to treat various internal and external parasite infestations. In addition to its antimalarial activity, it has anti-amoebic, anti-inflammatory, and antiviral effects (2-5). Ivermectin's another mechanism of action, inhibiting the in vitro replication of some positive single-stranded RNA viruses, has been the focus of attention (6-9). Various analytical methods, such as HPLC (10-14), electrospray ionization mass spectrometry (15), and various liquid or mass spectrometric or chromatographic methods, were used to determine ivermectin (16-19). A European Pharmacopoeia monograph was also made to analyze and identify several substances in samples of active pharmaceutical ingredients (20). In addition, an injectable product was analyzed with only one HPLC method for ivermectin (21). The method specified in the United States Pharmacopoeia is not preferred by quality control laboratories due to the long chromatographic study period. Therefore, there is still a great need for a sensitive, efficient and robust analytical method for the routine analysis of ivermectin in injectables and various end products.

Herein, we developed and validated new liquid chromatographic and spectrophotometric methods for ivermectin assay, including determining and identifying ivermectin in pharmaceutical products. These analytical

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Corresponding Author: Numan Taspinar, Uşak University, Faculty of Medicine, Department of Medical Pharmacology, Uşak, Türkiye **E-mail**: numan.taspinar@usak.edu.tr

methods are precise, accurate, linear, selective, and robust. Therefore, these newly developed and validated methods would suit quality control laboratories to analyze ivermectin in pharmaceutical products. Analysis of variance was used to compare the results of these analytical methods and their rest reliability was assessed by routine quality control analysis.

MATERIAL AND METHOD

Reagents

The reagents used in the research and the places they were supplied were as follows; Ivermectin from Sigma-Aldrich Chemie Gmb (St Louis, MO, USA); Bomectin® injection 500 mL (10 µg mL-1, ivermectin) from Elanco Australasia Pty Ltd (North Ryde, New South Wales, Australia); Stromectol (3 mg ivermectin) was from Merck Sharp & Dohme and Acetonitrile (HPLC grade) was from Sigma-Aldrich Chemie GmbH. Merck Millipore (Bedford, MA, USA) was used for ultrapure water.

Analytical Instruments and Conditions

HPLC analyzes were performed with Agilent 1260 system (Palo Alto, CA, USA) using a C18 (4.6 mm x 250 mm, 5.0 µm) column at 25°C room temperature. This system consisted of software, quad pump, UV detector and sample collector. Chromatographic detection was performed at 245 nm. The mobile phase measurements were performed at a flow rate of 1.2 mL/min and an injection volume of 20 µL, and the solution used for the measurements contained acetonitrile (20/80, v/v) and ultrapure water.

A Shimadzu UV 1800 dual-beam (Shimadzu, Kyoto, Japan) spectrophotometer with UV-Probe software and a 1 cm quartz cuvette was used for ultraviolet spectrophotometric analysis. Standard solutions were scanned in a UV spectrophotometer to determine the λmax value in the 200-400 nm range, and measurements were obtained against methanol in a blinded manner. The amount of ivermectin was determined and the absorbance values of the standard solution were measured at a wavelength of 245 nm and recorded. It has been shown that ivermectin absorbance values at this wavelength are proportional to standard mixture concentrations.

Preparation of Sample and Standard Solutions

Ivermectin standard solutions: It was dissolved by adding 25 mg of ivermectin reference standard and 20 mL of methanol to dissolve into a 50 mL volumetric tube. Ultrapure water was added to the tube until the total volume was 50 mL. This 500 µg/mL stock standard mix was then diluted with ultrapure water in ten increments to obtain six different concentrations of 10-60 µg / mL standard mix.

Ivermectin sample solutions: Powder tablets equivalent to 25 mg ivermectin raw material and/or 25 mg ivermectin were added to a 50 mL measuring bottle by adding 20 mL methanol and dissolved. The total volume was made up to 50 mL using methanol. Four milliliters of this solution were taken and diluted with methanol in a 50 mL measuring flask to yield 40 µg/mL ivermectin solution.

Validation

The optimized chromatographic and spectrophotometric methods are fully validated following protocols specified in ICH guidelines Q2(R1) to validate analytical methods (22). Parameters such as linearity, stability, sensitivity and system suitability tests were measured for validation purposes.

Linearity: Calibration curves of ivermectin prepared in 6 different densities of 10-60 ug/mL were plotted concentration versus peak area in triplicate (the chromatographic method and concentration versus absorbance value) for the spectrophotometric method. The evaluation of the linearity of the samples was made by regression analysis using the least squares method.

Precision: For six tablet samples (n = 6) at a concentration of 40 µg/mL, intraday precision analysis was performed by UV and HPLC methods. To determine the between-day precision values, measurements were made on three sequential days (n = 18) and the ivermectin content and relative standard deviations (RSD) values were calculated.

Accuracy: The accuracy of the methods was determined by recovery studies at three levels (80%, 100% and 120% of test concentration). This was done by analyzing a sample of known concentration and comparing the measured value with the "real" value. A well-characterized sample solution (40 µg/mL of ivermectin) was used. Samples were prepared in triplicate for each concentration, analyzed by UV and HPLC methods, and recovery percentages were calculated.

Specificity: 40 µg/mL ivermectin sample solution prepared according to the procedure was injected into the chromatographic system to identify possible interfering peaks.

The wavelength range of 200-400 nm was used for spectrophotometric analysis of ivermectin samples. As a result of the analyzes, the interference bands were observed at a wavelength of 245 nm. The spectral peak purity of ivermectin obtained as a result of the analyzes was measured with a diode array detector and ultraviolet spectra were evaluated.

Limits of Quantitation and Limits of Detection: The LOQ and LOD were used to evaluate the sensitivity of both chromatographic and spectrophotometric methods. LOD and LOQ values were calculated according to the formulation in the 1st and 2nd equations. The slope of the calibration curve and the standard deviation of the y-intercept were used in the calculation.

LOD = 3.3xσ/S (1)
LOQ = 10xσ/S (2)

S: the slope of the calibration curve
σ: standard deviation of y-intercept

Analysis of Marketed Formulations

Stromectol® tablet samples containing ivermectin and Bomectin injectable solution were prepared as described in the previous sections and analyzed using HPLC and UV methods. Ivermectin contents were determined using these two methods. ANOVA-Tukey test was used to determine the significance levels and P<0.05 was considered significant.

Comparative Analysis

Both analytical methods were used in these formulations. Evaluation of recovery percentages was done using F and t-test.

Stability of Solutions

The results of the reference standard solutions over 24 hours were used for stability assessment. Standard solutions were used for stabilization analyzes at room temperature (25°C) protected from light.

RESULTS

Method Development

Various preliminary studies were carried out to optimize the conditions for ivermectin quantification for chromatographic method development. First, only ultrapure water was tested without the use of organic modifiers and the results were recorded. Different ratios of acetonitrile solutions were used for optimum conditions. A good ivermectin peak and symmetry were determined at 80% acetonitrile ultrapure ratio.

Finally, it was determined that the mobile phase consisting of ultra-pure water and acetonitrile (20:80, v/v) provides more powerful theoretical plates (>7.000) and a peak queuing factor (<1.0). Mobile phases between at different flow rates (0.8–1.5 mL min-1) and containing organic solvents and ultrapure water at different pH ranges were tested. The best chromatographic conditions were achieved using an isocratic mobile phase comprising ultra-pure water (pH = 7.0)-acetonitrile (20/80, v/v) at a flow rate of 1.2 mL min−1 on an Agilent Extend-C18 (4.6 mm × 250 mm, 5.0 µm) that was kept at 25°C. In addition to being economical, the analysis was carried out at 25°C, which offers advantages such as increased column efficiency, low column pressure, and good chromatographic peak shape.

The eluent was monitored using a UV detector set to 245 nm. The ivermectin retention time in these chromatographic conditions was 12.54 minutes (Figure 1). Tablet samples and injection forms were analyzed for 1 hour to ensure that no matrix components remained in the column at the specified conditions. However, analyzing more than 15 minutes will both prolong the analysis time and increase the cost. No overlapping peaks were observed in the analysis of the samples during consecutive 15-minute analyzes. Therefore, the analysis time was set as 15 minutes.

The UV spectrum of ivermectin in the 200-400 nm range was evaluated (Figure 2). Ivermectin has shown sufficient molar absorptivity at a wavelength of 245 nm. The 245 nm wavelength showed higher selectivity for potantial interfering composites in the samples and therefore the 245 nm wavelength was chosen for detection.

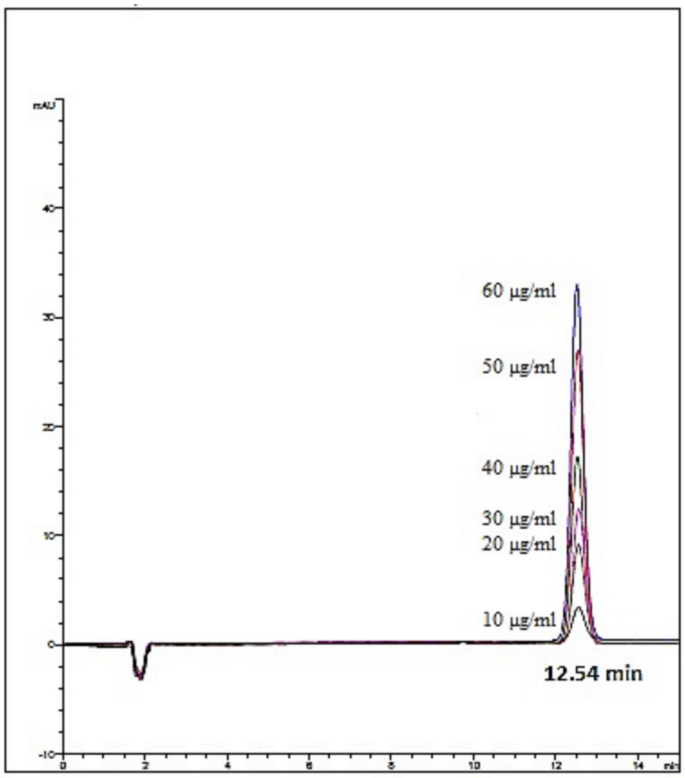

Figure 1. Overlay chromatogram obtained for ivermectin standard solutions (10-60 µg/ml, Retention time: 12.54 min.)

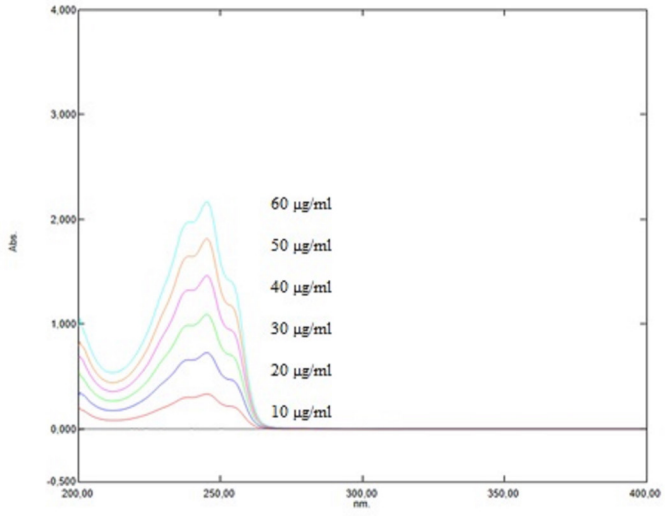

Figure 2. Overlay spectrum of ivermectin standard solutions (10-60 µg/ml)

Analytical Validation

A linear relationship was observed between ivermectin concentrations and the responses of both UV and HPLC methods (Table 1). The regression coefficient (R2) was high in both methods (>0.999) and there was no important different in linearity in the analyzed range.

As a result of the analyses, % RSD values for both analytical methods were lower than 2.0 (table 2). This low is an indication that the sensitivity of the methods is good. Accuracy was investigated using the standard addition method. Both analytical methods of recovery were close to 100% and showed sufficient accuracy (Table 1).

Table 1. The linearity data obtained for ivermectin by both methods

Regression parameters	HPLC	UV
Regression coefficient (R^2)	0.9999	0.9997
Slope±standard error	11.6040±0.1300	0.0366±0.0003
Intercept±standard error	-6.1067±1.078	−0.0153±0.0133
Relative standard error (%)	1.1582	0.55
Concentration range (µg/mL)	10-60	10-60
Number of points	6	6

Table 2. Validation parameters of the evaluated methods

Validation parameters	HPLC	UV
Intra-day precision, (RSD %, n = 6)	0.46	0.66
Inter-day precision, (RSD %, n = 18)	0.67	0.62
Accuracy, (mean recovery, %, n = 9)	99.62	98.95
LOD (µg/mL)/LOQ (µg/mL)	0.80/2.30	2.90/8.90
Concentration range (µg/mL)	10-60	10-60
Number of points	6	6

In the specificity analyzes performed by HPLC method, the ivermectin peak purity was greater than 99% in the sample mixture chromatograms. These results showed that the other compounds diverged from the main peak. No interference peaks were detected in the chromatogram obtained from the tablet excipient mixture used in the analysis of the retention time of ivermectin.

Absorption bands at 245 nm were not observed in the absorbance spectrum obtained from the tablet excipient mixture in methanol in quantifying the UV method. Therefore, this wavelength was shown to be selective for ivermectin in the UV method.

LOD and LOQ values for both methods were calculated as 0.80 and 2.30 µg/mL, respectively (Table 2). In the UV analysis at a wavelength of 245 nm, the absorbance value of the ivermectin standard solution at a concentration of 2.90 µg/mL was found to be 0.0908. Therefore, this concentration is set as the detection limit. The absorbance value of ivermectin standard solution at a concentration of 8.90 µg/mL at a wavelength of 245 nm was measured as 0.3104 (Table 2). Therefore, this concentration is set as the quantitation limit. According to the results obtained, it is proved that the HPLC method is a more sensitive method that allows determining the amount of ivermectin at concentrations about four times lower than the UV.

Marketed Formulations Analysis

The validated chromatographic and spectrophotometric methods were applied to the analysis of ivermectin in raw material, Stromectol tablets, and Bomectin injection solution (Table 3). As a result of the statistical analysis, no significant difference was observed between the methods in the ANOVA test for tablets and injectable solutions of ivermectin. Tukey's multiple comparison test showed that the means obtained by these methods for raw material analysis were statistically equivalent ($p<0.05$). Tukey test for analysis of tablets revealed statistical equivalence ($p<0.05$) between HPLC and UV averages.

Table 3. Ivermectin contents in raw material, tablet sample, and the injectable solution obtained by HPLC, and UV (n=12)

Samples	Ivermectin content (%) ± SD	
	HPLC	UV
Raw material	99.82±0.27	99.47±0.42
Tablet	98.94±0.53	98.03±0.65
Injectable solution	99.22±0.36	99.17±0.51
F-testi	0.31/0.52	
$F_{calculation}/F_{table}$		
t-testi	1.58/2.81	
$t_{calculation}/t_{table}$		

[a]SD = Standard deviation

Even though spectrophotometric analysis assessed degradation products or related chemicals with comparable chemical structures, potential interactions in raw material studies were not determined in any of the evaluated methods. It was observed that the chromatographic method is the most sensitive and selective method. This method can be successfully utilized for the determination of the amount of ivermectin. However, the time and expense of analysis cannot be overlooked. The spectrophotometric approach is more cost-effective and needs less analytical time while also being simple to apply. Because ivermectin is such a widely used antiparasitic drug, it is critical to develop and validate, reliable and straightforward procedures to verify the quality of raw materials and pharmaceutical formulations on the market today.

Statistical Comparison of Methods

As a result of the analyzes performed, the data obtained from both methods were statistically evaluated at the 95% confidence interval using F and t tests, and no significant difference was observed in either method (Table 3).

Table 4. Standard solution stability (n=3, 50 µg/mL)

Time period, h	Peak area	Avg. peak area	SD	RSD, %	Retention time, min	Avg. retention time, min	SD	RSD, %
0	573.0	573.2	0.20	0.027	12.553	12.547	0.018	0.142
	573.3				12.561			
	573.2				12.527			
24	573.5	572.8	0.60	0.109	12.536	12.535	0.018	0.144
	572.3				12.517			
	572.6				12.553			
48	572.4	572.7	0.30	0.053	12.532	12.534	0.006	0.045
	572.6				12.529			
	573.0				12.540			

It is obvious that both methods can be used for the determination of ivermectin in different pharmaceutical forms.

Stability of Standard Solutions

Standard solution injections were made into the HPLC system at eight-hour intervals, stability measurements of the standard solutions to be used as reference for 24 hours were made, and the retention times and peak areas were recorded. RSD % values were measured as 0.144% for the retention time and 0.109 for the peak area (Table 4). In addition, no significant change was detected in the active substance in the standard solution.

DISCUSSION

Analysis of different pharmaceutical forms of ivermectin was evaluated by two different methods (HPLC, UV spectrophotometric).

The results obtained from the developed analytical methods were statistically verified. The results showed that the spectrophotometric and liquid chromatographic methods were linear, precise, accurate, robust and durable with RSD < 1.00%, and the recovery percentage was within the specified limits (98-102%). A statistical comparison of these analytical methods was then made and the results of both methods showed no significant difference. At the 95% confidence interval, there was no statistically significant difference between the two techniques. The linearity data obtained for ivermectin by both methods, the validation parameters of the evaluated methods, the ivermectin contents in the raw material, tablet sample and injectable solution obtained by HPLC and UV, statistical comparison and standard solution stability results are given in Tables (I-V). In addition, the overlay chromatogram obtained for ivermectin standard solutions and the overlay spectrum results of ivermectin standard solutions are shown in Figures (1-2). The results of the analysis were explained in detail in the results section. The UV spectrophotometric method is more advantageous than the LC chromatographic method, as it is inexpensive, takes less time, and does not require detailed procedures and processes. However, according to the statistical comparisons, the results of the LC chromatographic method are more precise and accurate than the UV spectrophotometric method.

CONCLUSION

HPLC and UV spectrophotometric methods have shown that they are both adequate to determine the amount of ivermectin in raw materials, tablets and injectable solutions, and reliable results were obtained. No interfering absorption bands were observed in the UV spectrophotometric method at a wavelength of 245 nm, and no interference peaks were detected during the retention time of ivermectin in the LC chromatographic method. These methods are quick and simple. It can be used successfully in quality control analyzes to measure and identify the amount of ivermectin in commercially available preparations.

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Ethical approval: *Ethical approval is not required as no living material was used in the study.*

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