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RESEARCH ARTICLE

Development and Validation of an HPLC Method for Simultaneous Determination of Miconazole Nitrate and Chlorhexidine Digluconate in Chitosan-Based Gel Formulations

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SUMMARY

Miconazole nitrate (MN) and chlorhexidine digluconate (CHX) are the commonly used antimicrobials for topical treatment of dermal infections. Combination of antimicrobials has been investigated to enhance the efficacy of the treatment. Gel formulations based on bioadhesive polymers are preferred for delivery of these drugs. Chitosan is a promising bioadhesive polymer due to its penetration enhancing, antimicrobial and tissue healing properties. Yet, most of the gel-based formulations present analytical challenges during testing the drug content. It was aimed to develop an HPLC method for simultaneous determination of MN and CHX in chitosan-based gel formulations. Different solvent combinations were investigated for extraction of drugs from the gels. HPLC conditions such as mobile phase, flow rate, run time, column temperature and wavelength were explored. The method was validated according to ICH guideline Q2(R1). MN and CHX were extracted in solvent composition same with the mobile phase. The method was employed on ACE-C8 column at 40°C by isocratic elution using the mobile phase consisting of methanol:phosphate (75:25 v/v) buffer (containing triethylamine). Flow rate was 1 mL/min. The drugs were detected at 254 nm (CHX) and 230 nm (MN). Linearity was obtained between 5 to 80 µg/mL for both drugs. LOD and LOQ obtained for CHX were 1.61 and 4.87 µg/mL, for MN: 1.06 and 3.21 µg/mL, respectively. A new validated HPLC method was developed for simultaneous determination of CHX and MN in chitosan-based gels, with 98 to 102% recovery, without any interference with the excipients.

Key Words: *HPLC method, simultaneous analysis, miconazole nitrate, chlorhexidine digluconate, chitosan gel, validation*

Kitosan Bazlı Jel Formülasyonlarında Mikonazol Nitrat ve Klorheksidin Diglukonatın Eşzamanlı Tayini için Bir HPLC Yönteminin Geliştirilmesi ve Validasyonu

ÖZ

Mikonazol nitrat (MN) ve klorheksidin diglukonat (CHX), dermal enfeksiyonların topikal tedavisi için yaygın olarak kullanılan antimikrobiyallerdir. Tedavinin etkinliğini arttırmak için antimikrobiyallerin kombinasyonu araştırılmıştır. Bu ilaçların taşınması için biyoadezif polimer bazlı jel formülasyonları tercih edilmektedir. Kitosan, penetrasyon arttırıcı, antimikrobiyal ve doku iyileştirici özellikleri nedeniyle umut verici bir biyoadezif polimerdir. Jel bazlı formülasyonların çoğu, henüz ilaç içeriğinin test edilmesi sırasında analitik zorluklar göstermektedir. Kitosan bazlı jel formülasyonlarında MN ve CHX'in eş zamanlı tayini için bir YBSK yönteminin geliştirilmesi amaçlanmıştır. Jellerden ilaçların ekstraksiyonu için farklı çözücü kombinasyonları incelenmiştir. Mobil faz, akış hızı, çalışma süresi, kolon sıcaklığı ve dalga boyu gibi YBSK koşulları incelenmiştir. Yöntem, ICH kılavuzu Q2(R1)'e göre valide edilmiştir. MN ve CHX, mobil faz ile aynı çözücü bileşiminde ekstrakte edilmiştir. Yöntem, metanol:fosfat (75:25 v/v) tamponundan (trietilamin içeren) oluşan mobil faz kullanılarak izokratik elüsyon ile 40°C'de ACE-C8 kolonunda geliştirilmiştir. Akış hızı 1 mL/dk'dır. İlaçlar 254 nm'de (CHX) ve 230 nm'de (MN) tespit edilmiştir. Her iki ilaç için de 5 ile 80 µg/mL arasında doğrusallık elde edilmiştir. CHX için elde edilen LOD ve LOQ sırasıyla 1.61 ve 4.87 µg/mL, MN için 1.06 ve 3.21 µg/mL'dır. Kitosan bazlı jellerde, yardımcı maddelerle herhangi bir etkileşim olmaksızın %98 - 102 geri kazanım ile CHX ve MN' nin eşzamanlı tayini için yeni bir valide YBSK yöntemi geliştirilmiştir.

Anahtar Kelimeler: *YBSK yöntemi, eşzamanlı analiz, mikonazol nitrat, klorhekzidin diglukonat, kitosan jel, validasyon*

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INTRODUCTION

Chlorhexidine digluconate (CHX) is a bactericidal biguanide compound with broad-spectrum antibacterial and antifungal activity (Greenstein, 1986; Paulson, 2002; Kampf, 2018) . It is widely used both in human and veterinary medicine as an antimicrobial agent (Guaguère, 1996; Sarkiala-Kessel, 2012; Aronson, 2016; Brookes, 2020). There are currently numerous commercially available preparations of CHX in solution, tablet, aerosol, ointment, cream, lozenge, cloth, sponge and swab forms containing CHX at different concentrations (Silvestri, 2013; "Facts about Chlorhexidine Gluconate," 2017; "What Is Periochip," 2017; Hoang, 2021). CHX is commonly used as topical antiseptic and antimicrobial agent for wound cleansing and wound healing as well as for treatment of oral infections (Bouckaert, 1993; Rawlings, 1998; Şenel, 2000; Main, 2008; Atiyeh, 2009). CHX is positively charged and freely soluble in water (Mohammadi, 2008; Zeng, 2009).

Miconazole nitrate (MN) is an imidazole group drug used against fungal infections and gram-positive bacterial infections (Sawyer, 1975). MN has been widely used in human and veterinary medicine in treatment of super candidiasis and dermal infections, dermatophytosis and pityriasis versicolor through topical (Rochette, 2003; Frymus, 2013), vaginal (Kenechukwu, 2018; Salah, 2018), buccal (Cartagena, 2017; Tejada, 2018), oral (Dimopoulou, 2015) and parenteral (Wade, 1979) administrations. MN is a positively charged compound with 6.7 pKa value and very slightly soluble in water, methanol and alcohol (Al-Badr, 2005; *Martindale: The Complete Drug Reference*, 2009; Qushawy, 2018). The combination of MN with CHX has been shown to exert synergistic effect against numerous bacteria (Perrins, 2003; Mueller, 2008; Nenoff, 2017). Further, presence of the combination of ethylene diamine tetra acetic acid (EDTA) and hydroxymethyl aminomethane (Tris) has been shown to increase the sensitivity of the cell wall of the microbe to microbials (Guardabassi, 2010; Ghibaudo, 2016; Stojanov, 2018). In order to achieve successful topical formulations for delivery of antimicrobial agents, it is important to provide retention of the system on the application site for desired period of time and drug release in a prolonged fashion. Chitosan is a cationic biopolymer which is widely investigated for topical delivery of antimicrobials due to its bioadhesive and penetration enhancing properties as well as for its bioactive properties such as antimicrobial and wound healing (Şenel, 2010; Şenel, 2020). The most preferred form among the developed chitosan-based formulations are gels. However, most of the gel-based formulations present analytical challenges during testing the drug content. These products generally require burdensome extraction and sample preparation procedures. Especially, if there are more than one drug in the formulation the assay becomes more complicated.

Numerous analytical methods such as UV spectrophotometry, high-performance liquid chromatography (HPLC), etc., have been reported for precise quantification of MN (Heneedak, 2012; Belal, 2012; Ei, 2016; Maha Mohamed Abdelrahman, 2017; Eticha, 2018) or CHX (Borissova, 1997; Havlíková, 2007; Abtheen, 2008; Másquio Fiorentino, 2010; Chiapetta, 2011; Maha M. Abdelrahman, 2016; Işık, 2018) in the pharmaceutical dosage forms. However, due to the physico-chemical properties of MN and CHX, when incorporated together in a formulation the analytical methods are affected by interaction between the two drugs. Separation and retention of a polar and a non-polar compound by the same stationary phase can be a useful approach for simultaneous analysis of these compounds. An HPLC method developed for simultaneous determination of chlorhexidine, miconazole, clobetasol and neomycin in a cream formulation was reported by Kumar et al. (Kumar, 2017). In this method, a mixture of 20 mM phosphate buffer (pH 6.6) and acetonitrile at a ratio of 65:35 was used as the mobile phase, at 1 mL/min flow rate. Retention times for chlorhexidine and miconazole in the cream formulation were 4,927 and 5,606 min, respectively.

In this study, we aimed to develop and validate an HPLC method for simultaneous determination of MN and CHX in chitosan-based gel formulation, which we have developed for topical treatment of dermal infections.

MATERIAL AND METHODS

Materials

Miconazole nitrate was generously provided by IE Ulagay-Menarini Group (Turkey). Chitosan was generously provided by Koyo Co., LTD Japan. Chlorhexidine digluconate, Tris base (T6066), EDTA (E-5134) and Tween 80® (Cas no: 9005-65-6) were purchased from Sigma-Aldrich (Germany). Tween 20® was purchased from BDH Laboratory Supplies Poole, England and propylene glycol (Ph. USP Grade) from Merck Millipore (Germany). All other chemical reagents were of analytical grade.

Formulation Development

Chitosan gel was prepared at 3% (w/v) concentration in 2% v/v acetic acid. 2% w/v CHX and 2% w/v MN were incorporated into the gels. Tween 20® and Tween 80® were used as surfactants, propylene glycol and ethanol were used as co-solvents. Tris-EDTA (16:1) was also incorporated into the gels to enhance the antimicrobial activity (Türkmen, 2022).

Instrumental Conditions

HPLC measurements were performed on the Prominence LC-20A Modular HPLC System (Shimadzu, Japan). HPLC sample analysis and data collecting were conducted using LabSolutions software. The HPLC system consisted of a degasser (DGU-20A5), a pump (LC-20AT), an auto sampler (SIL-20A HT), a column oven (CTO-10AS VP). UV detection was performed at SPD-M20A (Photodiode Array Detector-UV-Vis Detector). For simultaneous determination of MN-CHX in chitosan-based gel formulations, different HPLC conditions such as mobile phase, flow rate, run time, column temperature and wavelength were investigated (Table 1). The HPLC conditions at the highest yield were determined as summarized in Table 2.

Table 1. Chromatographic conditions investigated

Mobile phase	Column	Ratio of mobile phase	Elution type	Flow rate (mL/min)	Run time (min)	Wavelength (nm)	
						CHX	MN
Methanol: 20 mM pH 3.0 phosphate buffer (0.1 % triethylamine)	ACE® C18 (250 x 4.6 mm, 5 µm)	80:20	Isocratic	1	30	254	230
Methanol: 20 mM pH 6.9 phosphate buffer (0.2 %TEA)	ACE® C8 (150 × 4.6 mm, 5 µm)	78:22	Isocratic	0.8 and 1	25	210 220 230 240 254 260	210 220 230 240 254 260
		78:22 - 85:15 78:22 - 82:18 78:22 - 80:20 78:22 - 75:25 78:22 - 72:18	Gradient	1	25	254	230
		75:25 78:22 80:20	Isocratic	1 and 1.2	25	254	230

Table 2. HPLC conditions of the developed method

Column	ACE C8 Column (150 mm x 4.6 mm, 5 µm)
Flow rate	1 mL/min
Wavelength	230 nm (MN), 254 nm (CHX)
Temperature	40 °C
Mobile phase	Methanol:20 mM pH 6.9 phosphate buffer (0.2% triethylamine) (75:25)
Injection volume	20 µL

Preparation of mobile phase

3.56 g of sodium phosphate dibasic dihydrate was weighed and dissolved in purified water and completed to 1000 mL. 2 mL of triethylamine (TEA) solution was added to the buffer solution at 0.2% v/v concentration. pH was adjusted to 6.9 by adding 5 M ortho-phosphoric acid. The pH of the mobile phase was measured pH meter (HANNA® Instruments, USA). The final buffer solution was filtered using mixed cellulose (CA-CN) membrane disc (diameter: 47mm; pore size: 0.22 µm) (Lubitech Technologies Ltd, China) and degassed for 30 min prior to use. Mixture of methanol: 20 mM pH 6.9 phosphate buffer (containing 0.2% v/v TEA) solution at different ratios (80:20, 78:22 and 75:25 for isocratic elution; 78:22-85:15, 78:22-82:18, 78:22-80:20, 78:22-75:25 and 78:22-72:18 for gradient elution) was prepared as the mobile phase. Methanol: buffer solution at 50:50 ratio was used for dilution of the gels and standard solutions.

Extraction procedure of the CHX and MN from gels

For extraction of both drugs from the gel, after trying different solvent systems, the most suitable solvent composition was found to be methanol: pH 6.9 phosphate buffer (0.2% TEA) at 75:25 v/v ratio, which is also the mobile phase. The gels were diluted in the extraction solvent and centrifuged at 8500 rpm for 10 min. The supernatant was withdrawn and diluted with mobile phase and injected into HPLC system.

System Suitability Test

System suitability test was performed to show that the system and developed method provides acceptable quality data. For this purpose, % RSD values of retention time and peak area, tailing factor parameters were determined using a standard solution at 80 µg/mL concentration for both drugs.

Method validation

The method was validated according to the International Council for Harmonization (ICH) guideline, ICHQ2(R1) ("Validation of Analytical Procedures: Text and Methodology Q2 (R1)", 1995), determining the parameters such as specificity, selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision and stability.

Specificity

Specificity was evaluated to show the absence of interference with the inactive ingredients used in the formulations (analytical placebo). The placebo solutions were prepared containing Tris: EDTA, Tween 20, Tween 80, propylene glycol and ethanol in chitosan gel. Samples were analyzed in six replicates.

Selectivity

The ability to separate the drugs in the sample was demonstrated by assessing the resolution between the peaks corresponding to CHX and MN. For the selectivity of the method, the standard solution of CHX and MN at the same concentration (80 µg/mL) was prepared as given at 2.3.2 section and injected into HPLC.

Linearity

The linearity of the method was determined using different concentrations (5, 10, 20, 40 and 80.0 µg/mL) of MN and CHX. The linearity was conducted at the same concentration range (5 to 80 µg/mL) for CHX and MN. The calibration curves were obtained by plotting peak area versus concentration. The correlation coefficients were calculated and the linearity was determined by linear regression analysis. The tests were performed in six replicates.

Accuracy

Accuracy was measured as the percent of deviation from the nominal concentration. Standard solutions with accurate concentrations (10 µg/mL, 20 µg/mL and 40 µg/mL) were prepared in six replicates and injected into the system. The recovery percent (recovery %) and the percentage relative standard deviation (RSD %) were calculated for each concentration. Bias % was calculated using Equation 1.

Bias % = [(Measured concentration - theoretical concentration)/ theoretical concentration] x 100 (1)

Precision

To determine the precision of the method, repeatability (same day) and reproducibility (three consecutive days) was evaluated by analyzing the MN and CHX in standard solution prepared at different concentrations (10 µg/mL, 20 µg/mL and 40 µg/mL) with six replicates. RSD % was calculated for each concentration.

Ruggedness

The ruggedness of the developed method was investigated using two different analysts. Standard solutions of CHX and MN (n=5) at 20 µg/ mL concentration were prepared and analyzed separately by two different analysts and the results were compared statistically. The ruggedness was evaluated with two system suitability parameters with the retention time and the peak area.

Robustness

The robustness of the developed method was analyzed at different flow rates and temperatures. Standard solutions of CHX and MN at 20 µg/mL concentration were prepared and analyzed at different flow rates (1 and 1.2 mL/min) and temperatures (39°C and 40°C), and the results were compared, statistically.

Detection and Quantification Limits

LOD and LOQ are defined as the minimum concentration at which the analytes can be detected and quantified, respectively. The LOQ and LOD of the method were determined based on the standard deviation of the response and the slope using Equations 2 and 3. The slope was estimated from the calibration curve.

$$\boldsymbol{LOD} = 3.3 \times \frac{\sigma}{S} \quad (2)$$

$$\boldsymbol{LOQ} = 10 \times \frac{\sigma}{S} \quad (3)$$

σ: standard deviation of the response

S: slope of the calibration curve

Stability

The stability of standards solutions was investigated by reinjection of the samples at 0, 12 and 24 h and measuring recovery % of CHX and MN. Furthermore, the stability of CHX and MN in dissolution medium (pH 5.0 phosphate buffer containing 0.5% Tween 80) was evaluated at 37 °C for 6 h at 0, 3 and 6 h.

Statistical analysis

Statistical analysis of data obtained during method validation was performed to demonstrate validity of the analytical method. Calculation of the mean (or average), standard deviation, relative standard deviation, confidence intervals, and regression analysis was performed using software package, SPSS.

RESULTS AND DISCUSSION

Homogeneous and opaque whitish color gels were prepared with pH of 5.5, which is an appropriate pH for the maintenance of the stability of the drugs, MN and CHX (Türkmen, 2022). CHX solutions have been reported to be stable between the pH range of 5 to 8 and showing the highest antimicrobial activity within this range (Denton, 2001; Paulson, 2002). Similarly, MN is stable in the pH range of 5-8 (Ammara, 2018) and antifungal activity of MN is not changed in this range (Siegel, 1977).

Extraction of CHX and MN from gels

Amongst the different solvents and their combinations used for extraction of MN and CHX from the gels, the highest recovery % was obtained with methanol:20 mM pH 6.9 phosphate buffer (0.2% TEA) (50:50, v/v) which is also the mobile phase (Table 3). The % recovery results of the sample at 20 µg/mL in methanol:20 mM pH 6.9 phosphate buffer (0.2% TEA) (50:50, v/v) extraction solution are shown in Table 4.

Table 3. Extraction of MN and CHX from gels

Extraction Solution	Extraction Recovery % of CHX	Extraction Recovery % of MN
0.1% acetic acid containing 1% w/v sodium lauryl sulfate	27.9 ± 8.3	39.6 ± 2.3
Methanol	65.4 ± 4.5	71.7 ± 2.7
Methanol: water: acetic acid (90:9:1, v/v/v)	75.8 ± 0.9	80.9 ± 1.2
Methanol: 20 mM pH 6.9 phosphate buffer (0.2% TEA) (50:50, v/v)	99.8± 1.1	101.0 ±1.6

Table 4. The results of Recovery % (at 20 µg/mL)

	CHX	MN
Measured concentration (µg/mL)	19.98	19.54
	20.06	20.02
	19.79	19.5
	19.86	19.68
	19.62	19.79
	20.06	19.8
Mean Concentration (µg/mL) ± SD	19.9 ± 0.17	19.7 ± 0.19
Recovery %	99.47	98.6

Method Development

In our preliminary studies for simultaneous quantification of MN and CHX, a UV-spectrophotometric method based on the rule of absorbance additivity was tried; however no satisfactory results were obtained. Hence, it was decided to continue with an HPLC method. Firstly, a suitable column was selected. Uniform peak shapes and better separation were obtained with the C8 (150 mm x 4.6 mm, 5 µm) column. Further the pH condition was investigated and pH 6.9 was decided to be the most suitable pH. The column temperature was kept at 40 °C to obtain a shorter retention time, knowing that both CHX and MN are stable with temperature change ("Final Report on the Safety Assessment of Chlorhexidine/Chlorhexidine Diacetate/Chlorhexidine Dihydrochloride/Chlorhexidine Digluconate", 1993; Sahoo, 2016). Optimization of the mobile phase in HPLC separation is an essential step for the selectivity of the method and the retention time of the substances (Valkó, 1993; Samanidou, 2015). Hence, for mobile phase, the solvents were chosen taking the physico-chemical properties of the drugs, MN (hydrophobic) and CHX (hydrophilic, ionizable) into consideration. Due to the ionizable property of the drug, the pH of mobile phase can be one of the important variables in control of the retention in HPLC separation. The retention time of analyte is known to be affected by the pH changes of the mobile phase (Moldoveanu, 2017). Thus, buffers are widely used for the pH control of mobile phase (Lakka, 2019). Phosphate buffer at different pH (3.0 to 7.4) was investigated as the mobile phase for separation of CHX and MN. pH 6.9 was found to be the most suitable pH avoiding the noise peaks, which is also right pH for the stability of these drugs. TEA at 0.2 % v/v was added to the mobile phase to suppress the tailing of the peaks. Mixtures of methanol:water, methanol:phosphate buffer (pH 6.9), acetonitrile:water, acetonitrile:phosphate buffer (pH 6.9) at different ratios were investigated for separation of MN and CHX in the column at different flow rates to achieve short retention time and high separation efficiency for both CHX and MN (Table 5).

The standard solution prepared from the gel formulation containing CHX and MN was tested at different ratios of mobile phase, wavelengths and flow rates. The standard solution has been analyzed at a wavelength range of 210 to 260 nm with a mixture

of methanol:20 mM pH 6.9 phosphate buffer (0.2 % TEA) (78:22; v/v) as mobile phase and the acceptable system suitability parameters regarding of the chromatograms were obtained at 254 nm for CHX and 230 nm for MN.

The flow rate was changed from 0.8 mL/min to 1 mL/min to improve the column efficiency. Gradient elution with changing concentrations of methanol: buffer solution was analyzed. CHX was not completely eluted from the column with the gradient elution program of the mixture of methanol: 20 mM pH 6.9 phosphate buffer (0.2 % TEA) (78:22 - 85:15 v/v) at the end of the run time. Furthermore, the column efficiency of MN was found to be higher at 230 nm whilst the column efficiency of CHX was found to be low at 254 nm. Column efficiency was found to be >1500 with the gradient elution program of the mixture of methanol:20 mM pH 6.9 phosphate buffer (0.2 % TEA) (78:22 - 72:28 v/v). Further, isocratic elution with constant concentrations of methanol:20 mM pH 6.9 phosphate buffer (0.2 % TEA) was analyzed. 1 mL/min and 1.2 mL/min flow rates were tried to optimize the theoretical plate numbers. The highest theoretical plate numbers were reached in the mobile phase with a ratio of 75:25 v/v at a flow rate of 1 mL/min. A flow rate of 1.2 mL/min was not chosen due to insufficient improvement in the retention times of the peaks and the theoretical plate numbers and undesirable increase in the column back pressure. The well-defined separation of CHX and MN was achieved by isocratic elution at a mobile phase ratio of 75:25 with 1 mL/min flow rate. The retention time of CHX and MN was detected 15.87 min and 3.78 min, respectively (Figure 1 and Figure 2).

Table 5. Chromatographic conditions investigated

Mobile phase	Column	Ratio of mobile phase	Wavelength (nm)		Retention time (min)		Tailing Factor	
			CHX	MN	CHX	MN	CHX	MN
Methanol: 20 mM pH 3.0 phosphate buffer (0.1 % triethylamine)	C18 (250 x 4.6 mm, 5 µm)	80:20	254	230	3.08	17.23	4.71	1.19
Methanol: 20 mM pH 6.9 phosphate buffer (0.2 %TEA)	ACE® C8 (150 × 4.6 mm, 5 µm)	78:22	210		3.21		1.02	
			220		3.21		1.2	
			230		3.21		1.09	
			240		13.69		1.03	
			254		13.68		1.14	
			260		13.67		1.11	
		78:22 (0.8 mL/min)	210		3.906		1.273	
			220		3.906		1.13	
			230		3.906		1.135	
			240		17.573		1.073	
			254		17.565		1.074	
			260		17.579		1.089	
		78:22 - 85:15	254	230	21.58	3.18	1.24	1.18
		78:22 - 82:18			18.7	3.15	1.2	1.21
		78:22 - 80:20			17.53	3.17	1.27	1.22
		78:22 - 75:25			15.42	3.11	1.255	1.26
		78:22 - 72:28			14.64	3.28	1.271	1.22
		75:25	254	230	14.39	3.64	1.314	1.19
		78:22			16.57	3.28	1.33	0.72
		80:20			18.89	2.98	1.22	1.3

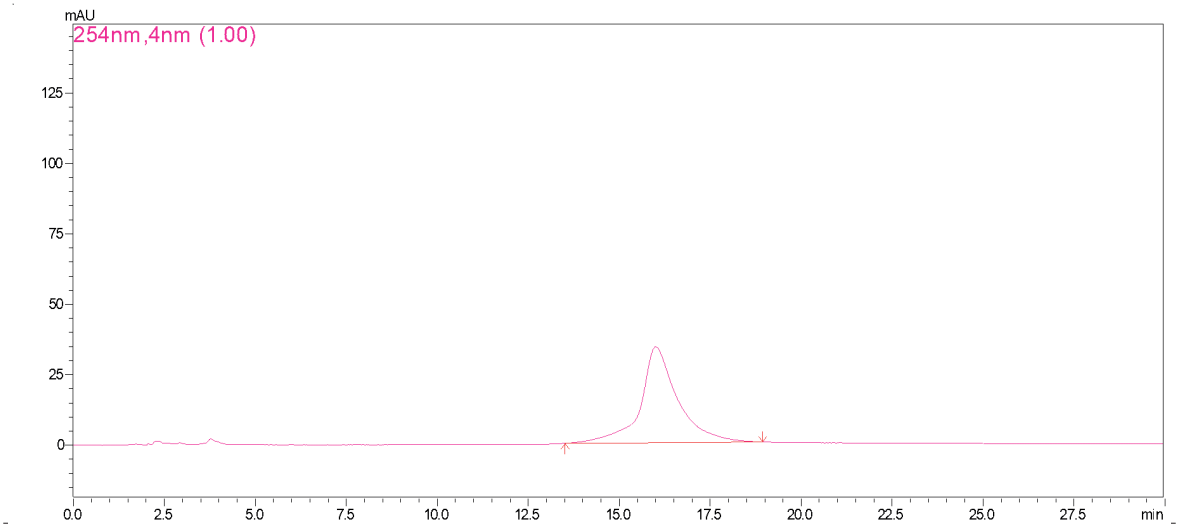

Figure 1. The chromatogram of the mixture of CHX (80 µg/mL) and MN (80 µg/mL) in mobile phase at 254 nm

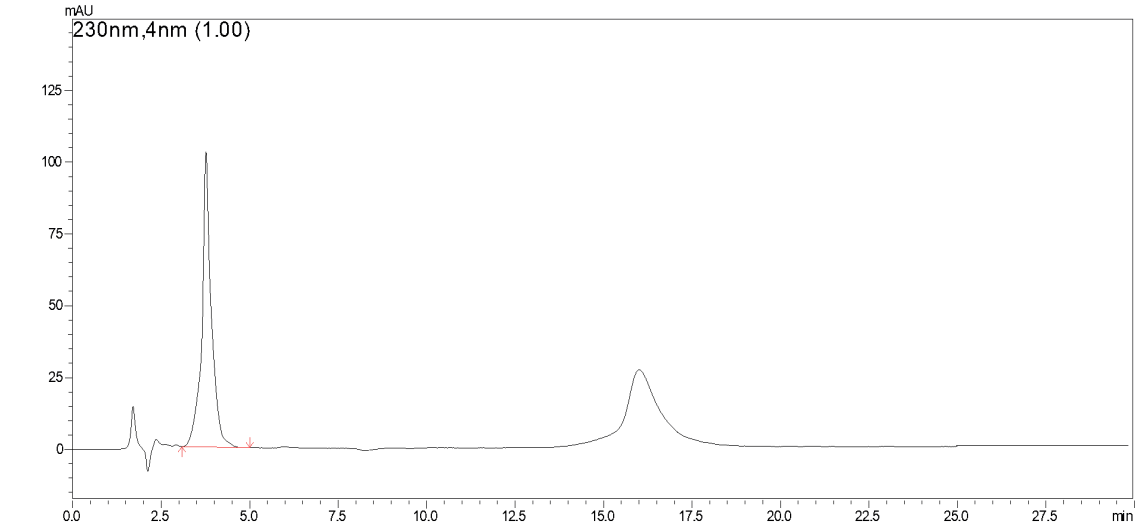

Figure 2. The chromatogram of the mixture of CHX (80 µg/mL) and MN (80 µg/mL) in mobile phase at 230 nm.

In conclusion, it was decided to use methanol:20 mM pH 6.9 phosphate buffer containing 0.2% TEA ratio as 75:25 (v/v) at 40 °C with 1 mL/min as flow rate to perform the analysis. Analysis was performed with wavelength at 254 nm for CHX and 230 nm for MN. The injection volume was chosen as 20 µL for all samples.

System Suitability

The system suitability results are summarized in Table 6. All parameters were shown to be in acceptable limits.

Table 6. System suitability results

Parameter	CHX	MN
% RSD of retention time	0.76	0.21
% RSD of peak area	0.1	0.09
Tailing factor (mean)	1.05	1.04

Method Validation

The developed method was validated in regard to selectivity, linearity range, accuracy, precision, sensitivity (LOD and LOQ) and stability according to the ICH guideline as stated in section 2.4.

Specificity and Selectivity

No interference between the drugs as well as between drugs and the inactive ingredients (Tween 20, Tween80, ethanol, propylene glycol, Tris-EDTA) was observed, indicating the selectivity of the developed method (Figures 3 and 4). Two separate peaks with good resolution and two different retention times were observed for MN and CHX.

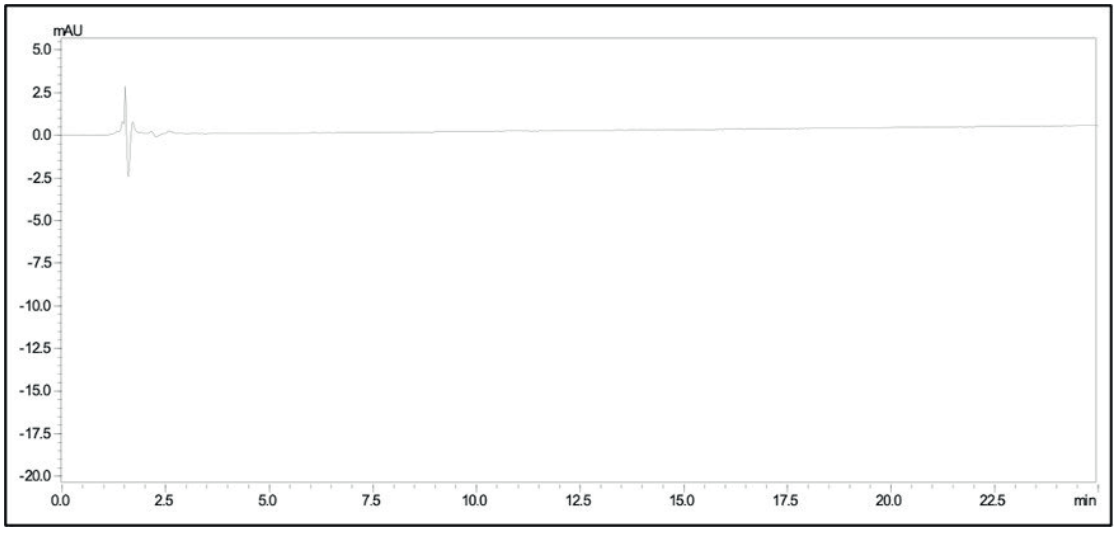

Figure 3. Chromatogram of placebo solution at 254 nm

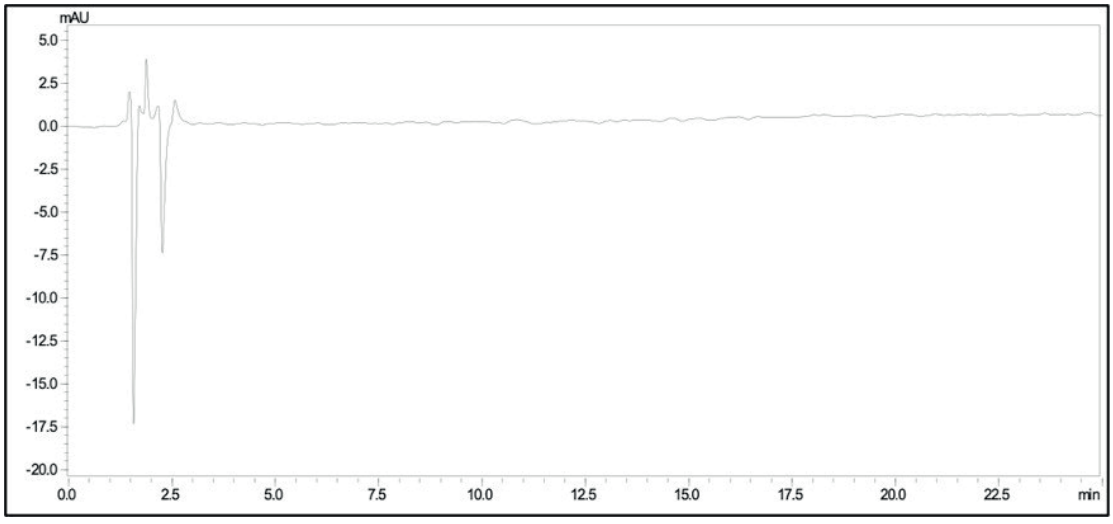

Figure 4. Chromatogram of placebo solution at 230 nm

Linearity

The linearity of the developed method was shown for both CHX and MN in the concentration range of 5 to 80 µg/mL with correlation coefficients of 0.9998 for CHX and 0.9999 for MN (Table 7, Figures 5 and 6).

Table 7. The results of linearity

	CHX	MN
Wavelength	254	230
Regression equation	y = 36394x - 89879	y = 25782x - 18982
Correlation coefficient (R^2)	0.9998	0.9999
Range	5 - 80 µg/mL	5 - 80 µg/mL

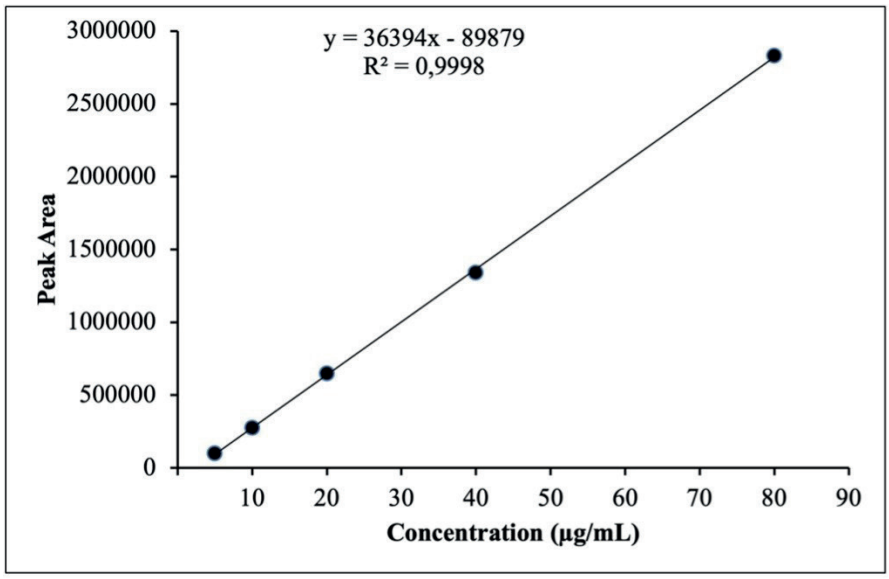

Figure 5. The calibration curve for CHX

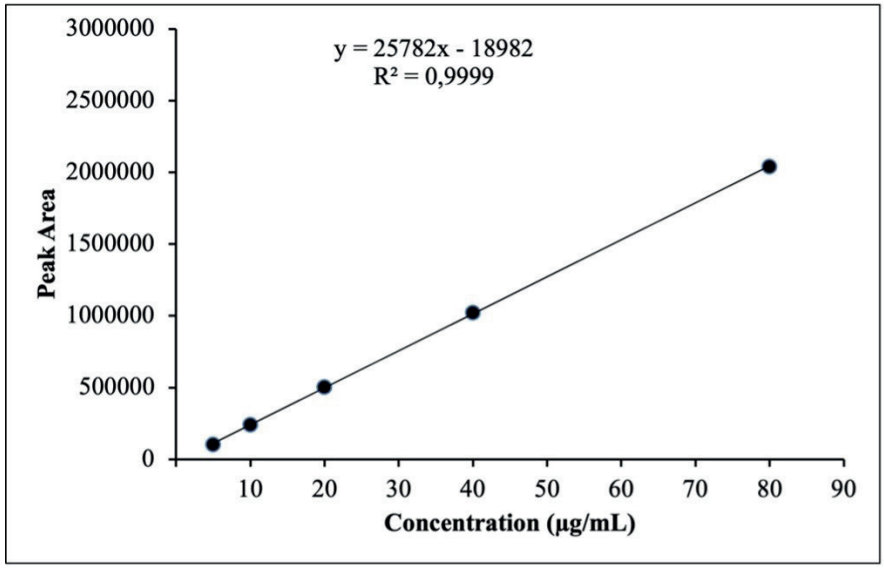

Figure 6. The calibration curve for MN

Accuracy and precision

% RSD values smaller than 1.5, recovery % larger than 98 % with very low % bias values were obtained with both intra- and inter-day analyses, indicating the precision and accuracy of the developed method (Table 8). The p-value for % recovery of CHX was 0.64 ($p>0.05$) and 0.8 ($p>0.05$) for MN according to the t-test results. There was no significant difference between intraday and interday results.

Table 8. Accuracy and precision of the developed method

	Theoretical conc. (µg/mL)	Intra-day				Inter-day (three consecutive days)			
		Measured conc. (µg/mL) ± SD	Precision RSD %	Accuracy Recovery %	Accuracy Bias %	Measured conc. (µg/mL) ± SD	Precision RSD %	Accuracy Recovery %	Accuracy Bias %
CHX	10	9.98±0.09	0.92	99.8	-0.2	10.1±0.1	0.77	100.6	0.6
	20	20.3±0.1	0.21	101.5	1.45	19.91±0.1	0.40	99.6	-0.4
	40	39.33±0.6	1.42	98.2	-1.75	40.81 ±0.11	0.29	102	2
MN	10	10.1±0.1	0.93	100.4	0.4	9.89±0.08	0.82	98.9	-1.1
	20	20.2±0.3	1.29	100.9	0.85	20.2±0.3	1.5	100.9	0.9
	40	40.3 ±0.4	0.96	100.8	0.85	40.7±0.6	1.46	101.7	1.7

Ruggedness

The % recovery results and %RSD of peak area and retention time obtained by two different analysts are given in Table 9 and Table 10. The results obtained were statistically evaluated with the t-test and there was no difference between two analysts (p >0,05). Moreover, % RSD values were found to be smaller than 1.48 indicating the ruggedness of the developed method (Table 10).

Table 9. The results of ruggedness by two different analysts

	CHX		MN	
	Analyst A	Analyst B	Analyst A	Analyst B
Measured concentration (µg/mL)	20.34	20.41	20.43	20.33
	20.26	20.36	20.27	20.26
	20.28	19.95	19.84	20.05
	20.22	20.33	19.88	19.62
	20.33	20.44	20.17	20.25
Mean Concentration (µg/mL) ± SD	20.2±0.05	20.3 ± 0.2	20,12±0,25	20.1±0.3
Recovery %	101.4	101.2	100.6	100.51

Table 10. The system suitability parameters of ruggedness results

	% RSD of retention time		% RSD of peak area	
	Analyst A	Analyst B	Analyst A	Analyst B
CHX	0.38	0.07	0.27	1.13
MN	0.14	0.17	1.29	1.48

Robustness

The robustness of the developed method was evaluated with different column temperatures and flow rates. The %RSD values of retention time at different conditions are given in Table 11. The % RSD values were smaller than 0,72. The robustness of the method has been shown (Table 11). The results obtained were statistically evaluated with the t-test and p >0,5 was obtained.

Table 11. The results of robustness by different chromatographic conditions

Chromatographic conditions	Value	CHX		MN	
		Retention time	% RSD	Retention time	% RSD
Column Temperature (°C)	39	14.49	0.32	3.88	0.04
	40	14.59	0.36	3.89	0.21
Flow rate (mL/min)	1	14.06	0.72	3.87	0.18
	1.2	15.25	0.53	3.77	0.24

Detection and Quantification Limits

LOD obtained for CHX and MN were 1.61 µg/mL and 1.06 µg/mL, respectively. LOQ for CHX and MN were 4.87 µg/mL and 3.21 µg/mL, respectively.

Stability

It was demonstrated that sample solutions were stable in mobile phase for 24h, with RSD% <1.3 and recovery % >98.5 for both MN and CHX. Furthermore, the recovery % was found to be >98 also in the dissolution medium (pH 5.0 phosphate buffer containing 0.5% Tween 80) (Table 12).

Table 12. The stability results (n = 6)

	Mobile Phase					Dissolution Medium				
	Theoretical conc. (µg/mL)	Time (h)	Measured conc. (µg/mL) ± SD	Recovery %	RSD %	Theoretical conc. (µg/mL)	Time (h)	Measured conc. (µg/mL) ± SD	Recovery %	RSD %
CHX	20	0	20.3± 0.1	101.5	0.2	80	0	80.2±0.78	100.3	0.97
	20	12	19.9± 0.2	99.5	0.9	80	3	79.4±0.9	99.2	1.12
	20	24	19.9± 0.2	99.9	0.8	80	6	80.4±0.7	100.5	0.83
MN	20	0	20.2± 0.3	100.9	1.3	80	0	80.9±0.7	101.2	0.88
	20	12	19.7 ± 0.2	98.6	0.9	80	3	80.2±0.5	100.2	0.59
	20	24	19.7 ± 0.1	98.5	0.2	80	6	78.5±0.2	98.1	0.26

The results of the validation showed that the HPLC method possesses significant linearity, specificity, selectivity, accuracy, precision, sensitivity, high efficiency and resolution, and no interference with the excipients used in the formulation.

Sample solutions were shown to be stable during analysis and the developed method was shown to be applicable to the sample solutions taken at dissolution studies without any stability problems.

CONCLUSION

We have successfully developed an HPLC method for simultaneous analysis of CHX and MN with short analysis time and high reproducibility, repeatability and sensitivity. Upto the authors knowledge, this is the first report in the literature for simultaneous analysis of CHX and MN from a chitosan-based gel formulation. Best chromatographic conditions were obtained with ACE® HPLC C8 column of 5 µm particle size (150 × 4.6 mm), with the mobile phase consisting of the mixture of methanol:20 mM pH 6.9 phosphate buffer (0.2 % TEA) (75:25 v/v), providing sufficient selectivity and sensitivity in a short separation time with acceptable peak characteristics, number of theoretical plates and acceptable resolution of MN and CHX, confirming the capability of the developed method. Furthermore, preparation of samples (extraction of MN and CHX from the gels and dilution) was also successful developed allowing recovery % >98. The developed method is suggested for simultaneous analysis of CHX and MN in gel formulations for quality control and in vitro tests to assure the quality and efficacy of the pharmaceutical preparations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Idea (SŞ), planning the development of HPLC analyses methodology for the active substances (SŞ, MÇ, ET and SP), the manuscript designing and editing (SŞ, MÇ, SP and ET), performing experiments (ET, SP), data interpretation (SŞ, ÇM, SP and ET), literature review (SŞ, ET and SP).

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