Printed in the Republic of Korea
ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 31 No. 2, 88-95, 2018
https://doi.org/10.5806/AST.2018.31.2.88

Simultaneous determination of fluorometholone and tetrahydrozoline hydrochloride in ophthalmic suspension by HPLC

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(Received February 3, 2018; Revised April 6, 2018; Accepted April 7, 2018)

Abstract A simple, sensitive, and selective high-performance liquid chromatography (HPLC) method was developed and validated for the simultaneous quantification of fluorometholone (FLU) and tetrahydrozoline hydrochloride (THZ) in combined ophthalmic formulations. The effects of several factors, including detection wavelength, buffer pH, buffer concentration, and organic solvent concentration, on the performance of the method were investigated. The performance of the developed method was validated in accordance with the requirements of the International Conference on Harmonization. The linearity of the calibration curves in the desired concentration range was high for both FLU and THZ (r^2 = 0.9999). The accuracy and precision of the method were determined by a recovery study at analyte concentrations of 80 %, 100 %, and 120 %. The recovery was 99.60–100.17 % for both analytes, with a relative standard deviation less than 1.0 % at any concentration level. The validated results indicate that the new HPLC method could be successfully applied for the simultaneous determination of FLU and THZ in ophthalmic suspensions.

Key words: HPLC, Fluorometholone, Tetrahydrozoline hydrochloride, Determination, Validation, Ophthalmic suspension

1. Introduction

Fluorometholone (FLU) (*Fig.* 1(a)) is chemically named as 9a-fluoro-11b,17a-dihydroxy-6a-methyl-pregna-1,4-diene-3,20-dione, and is a synthetic fluorinated glucocorticoid with anti-inflammatory and anti-allergenic properties. FLU is usually used in the treatment of allergies and inflammatory eye diseases, as well as skin disorders.[1]

Tetrahydrozoline hydrochloride (THZ) (*Fig.* 1(b)) is chemically named as 2-[(1*RS*)-1,2,3,4-tetrahydronaphthalen-1-yl]-4,5-dihydro-1H-imidazole hydrochloride. THZ is a sympathomimetic agent with α-adrenergic activity. Its primary application is for constricting

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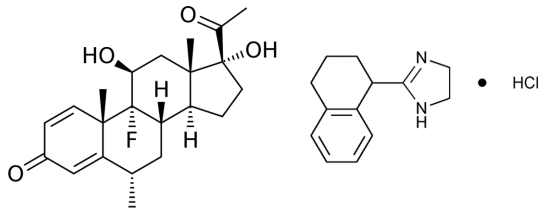


(a) (b)

Fig. 1. Chemical structure of (a) fluorometholone, (b) tetrahydrozoline hydrochloride.

conjunctive blood vessels, which decreases eye redness caused by minor ocular irritants.[2]

FLU and THZ are commonly used as ophthalmic dosage forms in the treatment of allergic and inflammatory eye diseases.[1,2] Due to their additive effects, a combination of FLU and THZ has been proven to be more effective than each individual drug alone.[3] Therefore, several combinations of these components are available on the pharmaceutical market for ophthalmic use.

From the review of literature, it was found that high-performance liquid chromatography (HPLC) methods are available for the determination of FLU and THZ, both individually and in combination with other drugs. To the best of our knowledge, most methods for the simultaneous determination of both compounds by HPLC still have some limitations. In particular, the peak retention times of THZ are short; therefore, their capacity factors are relatively small (less than 1.0).[4,5] Furthermore, several methods used different wavelengths to detect THZ and FLU,[4,6] which is not convenient or suitable for all instruments.

On the other hand, drug products with these combinations are not official in various pharmacopoeias, such as British Pharmacopoeia (BP 2016),[7] European Pharmacopoeia (EP 8.0),[8] United States Pharmacopoeia (USP 39),[9] and Japanese Pharmacopoeia (JP 17).[10] In addition to this, the Korean Pharmacopoeia (KP XI)[11] has a monograph named "Fluorometholone and tetrahydrozoline hydrochloride in ophthalmic suspension". However, the analytical method applied in this monograph is not convenient or efficient as each compound is quantified separately. The presence of FLU is determined by HPLC, while THZ content is quantified with ultraviolet-visible (UV) spectrophotometry.

Recently, there is growing need to replace inconvenient methods with more appropriate methods, as well as establish a simultaneous method to efficiently separate combined drugs, in the pharmaceutical industry.

Hence, the objective of this study is to develop an HPLC method that is efficient, applicable, and affordable for the simultaneous determination of FLU and THZ in ophthalmic suspensions. To fulfil this aim, the effects of various factors under chromatographic conditions are investigated to determine an optimized assay method. Validation of the results is conducted in accordance with the International Conference on Harmonization (ICH)[12] and Korean Food and Drug Administration (KFDA) validation protocols.[13,14]

2. Experimental

2.1. Chemicals and reagents

The active components of the FLU and THZ, and ophthalmic suspensions were provided by Hanmi Pharmaceutical (Seoul, Korea). HPLC-grade methanol was obtained from Daejung Chemicals and Metals Co. (Siheung, Korea). Potassium dihydrogen phosphate was purchased from Duksan Pure Chemicals Co. (Ansan, Korea). Purified water was prepared in the laboratory. All other chemicals used were of analytical reagent grade.

2.2. Analytical instruments and chromatographic conditions

The experiments were conducted with an Agilent 1100 HPLC system that consisted of the following components: G1379A Degasser, G1312 Binary Pump, G1313 Auto-sampler, G1316 Colcom (Column Oven), and G1314AVWD Detector (Agilent Technology, Santa Clara, USA).

For intermediate precision validation, a Shimadzu HPLC system was used, which included a DGU – 20A5R Degasser, two LC – 20 AD pumps, SIL – 20A autosampler, SPD-20A UV – Vis Detector, CBM – 20A communication bus module (Shimadzu Corporation,

Kyoto, Japan), and CO-965 Column Oven (Jasco Corporation, Tokyo, Japan).

For the optimized HPLC conditions, an Aegispak C18-F column (150 × 4.6 mm I.D., 5 μm) was used for gradient elution by using a binary mixture of eluent A (20 mM potassium dihydrogen phosphate at pH 5.0 and methanol (9:1, v/v)) and eluent B (methanol) at a flow rate of 1.0 mL/min. Gradients started at 70 % of eluent A and 30 % of eluent B and later increased to 65 % of eluent B over 4 min. This ratio was then maintained for 5 min. Then, the ratio of eluent B was decreased linearly to 30 % over 1 min. Finally, this process finished with an isocratic elution step for 5 min. A detector wavelength of 220 nm was used to quantify both FLU and THZ.

2.3. Preparation of stock and standard solutions

Considering the solubility of both FLU and THZ in methanol and water, a mixture of water and methanol in the ratio of 50:50 (v/v) was used as a diluent to prepare the solutions.

A stock solution of FLU was prepared by dissolving 100 mg of FLU in 50 mL of methanol to obtain a 2000 μg/mL solution.

A stock solution of THZ was prepared by dissolving 25 mg of THZ in 50 mL diluent to obtain a 500 μg/mL solution.

For the standard solution, the above stock solutions were further diluted with a diluent to obtain a concentration of 200 μg/mL and 50 μg/mL for FLU and THZ, respectively.

2.4. Preparation of sample solution

A volume of well-shaken ophthalmic suspension containing the equivalent of 2 mg of FLU and 0.5 mg THZ was transferred to a 10 mL volumetric flask. Methanol was added to approximately 50% capacity of the flask. To completely dissolve both compounds, the content of the flask was vortexed for 1 min and sonicated for 2 min. This was then cooled to room temperature and diluted with diluent to the mark and mixed. A portion of this solution was filtered through a 0.45 μm membrane to obtain the sample solution.

2.5. Method validation

The FLU/THZ ophthalmic formulations that were used in this study, are only available as mixtures with 1 mg/ 0.25 mg per 1 mL of FLU and THZ, respectively. The developed method was validated in accordance with the ICH guidelines Q2(R1) with regard to system suitability, linearity, limits of detection (LOD), limit of quantitation (LOQ), precision, accuracy, and robustness.

The linearity of the proposed HPLC procedure was evaluated using different concentrations (25 – 300 %) of the analytes (200 μg/mL for FLU and 50 μg/mL for THZ). Linearity was estimated using the coefficient of determination (r^2) of regression lines from six repeated analyses within the desired concentration range. Detection and quantification limits were based on signal-to- noise ratio, 3:1 and 10:1, respectively from six repeated analyses.

Precision (relative standard deviation, RSD %) of the method was assessed by (1) six analyses in one day (intra–day), and (2) over three different days (inter–day), using standard solutions at concentrations corresponding to 80, 100, and 120 % of analytes (160, 200, 240 μg/mL for FLU and 40, 50, 60 μg/mL for THZ).

Accuracy was expressed as recovery rates that were evaluated using the standard addition method: three concentrations (160, 200, 240 μg/mL for FLU and 40, 50, 60 μg/mL for THZ) were added into a sample solution consisting of 200 μg/mL of FLU and 50 μg/mL of THZ. The experiments were performed in triplicate.

2.6. Application of the method

The following analytical method was applied to quantify the content of FLU and THZ in ophthalmic suspensions. The study was conducted on six samples that were prepared from an ophthalmic suspension (described above). The amount of FLU and THZ in sample was calculated as follows:

$$\text{Fluorometholone } (C_{22}H_{29}FO_4)\ (\text{mg}) = m_{(F)} \times \frac{A_{T(F)}}{A_{S(F)}}$$

$$\text{Tetrahydrozoline HCl } (C_{13}H_{16}N_2.HCl)\ (\text{mg}) = m_{(T)} \times \frac{A_{T(T)}}{A_{S(T)}}$$

where, $m_{(F)}$, $m_{(T)}$ (mg) are the amount of FLU and

THZ weighed, respectively; $A_{S(F)}$, $A_{S(T)}$ (mAU*s) are peak area of FLU and THZ in the standard solution, respectively; and $A_{T(F)}$, $A_{T(T)}$ (mAU*s) are the peak area of FLU and THZ in the sample solution, respectively.

3. Results and Discussion

3.1. Examination of the compendial method

In KP XI, the compendial methods for FLU and THZ quantification in ophthalmic suspensions are HPLC and UV spectrophotometry, respectively. In preliminary experiment, chromatographic conditions of FLU following KP XI were applied to simultaneously determine both compounds. An Aegispak C18-F column (150 × 4.6 mm I.D., 5 μm) was used as the stationary phase, the flow rate was 1.0 mL/min, and the injected volume was 10 μL. The wavelength used for UV spectroscopy was 254 nm. The mobile phase consisted of methanol and water (60:40, v/v). Under these conditions the retention times for THZ and FLU were approximately 2 and 19 min, respectively. For THZ, the number of theoretical plates (N) was lower than 500 and the tailing factor was greater than 3. Therefore, the various factors that could affect the chromatographic performance were investigated with a focus on the retention time, column efficiency, and peak shape.

3.2. Development of the HPLC method

3.2.1. Selection of organic solvent concentration

In accordance with the pharmacopoeia such as USP 39, BP 2016, JP 17 and KP XI, methanol was selected as the organic solvent in this study. Its concentration was varied between 10-60 % (v/v), while the concentration of potassium dihydrogen phosphate buffer was maintained at 50 mM (pH 2.5). As the methanol content increased, the retention time of THZ and FLU decreased (*Table* 1). Due to the shortest retention time for both compounds, 60 % methanol was chosen for further experiments.

Table 1. Results of the investigation of mobile phase

Factor	Value	THZ				FLU			
		tR (min)	k'	N	TF	tR (min)	k'	N	TF
% methanol (%)	10	>45	-	-	-	>45	-	-	-
	20	13.20	6.32	5359.73	1.88	>60	-	-	-
	30	6.49	3.19	4257.02	1.63	>60	-	-	-
	40	3.93	1.17	3311.24	1.43	>60	-	-	-
	50	2.78	0.54	2676.97	1.42	28.88	15.03	8105.76	0.98
	60	2.26	0.31	2444.80	1.43	9.65	4.61	6691.82	1.03
pH of buffer	2.5	2.23	0.30	2102.87	1.42	11.00	5.39	6373.59	1.02
	3.0	2.20	0.38	2116.81	1.43	10.17	5.35	6293.63	1.03
	3.5	2.21	0.20	2113.87	1.45	9.94	4.39	6302.01	1.03
	4.0	2.24	0.28	2115.10	1.42	10.78	5.14	6306.17	1.03
	4.5	2.21	0.30	2120.15	1.44	9.97	4.86	6322.62	1.03
	5.0	2.25	0.31	2360.40	1.44	10.07	4.86	6338.25	1.03
Buffer concentration (mM)	0	1.82	0.14	1181.49	1.03	11.82	6.40	6499.00	1.02
	20	2.25	0.30	2212.88	1.54	10.58	5.14	6398.37	1.02
	40	2.26	0.31	2331.93	1.47	10.32	5.00	6322.42	1.03
	50	2.25	0.31	2360.40	1.44	10.07	4.86	6338.25	1.03
	60	2.39	0.38	2249.45	1.40	13.15	6.59	6682.78	1.01

tR (min): retention time.
k': capacity factor.
N: number of theoretical plate.
TF: tailing factor.

3.2.2. Selection of buffer pH

pH values of the buffer solutions differed among references.[3-5] In this study, a range of pH values from 2.5 to 5.0 were used to identify the most suitable pH, while the methanol content was fixed at 60 % (v/v). As can be seen in *Table* 1, the highest theoretical plate number was observed at pH 5.0, which was therefore selected as the buffer pH.

3.2.3. Selection of buffer concentration

Six different concentrations of phosphate buffer were tested to identify the optimal buffer with methanol content fixed at 60 % (v/v) and pH maintained at 5.0. As shown in *Table* 1, without phosphate buffer, the number of theoretical plates of THZ was approximately 1000, and FLU was retained for 12 min. When the buffer concentration increased from 20 to 60 mM, no significant difference in chromatographic parameters was observed. In order to prevent potential buffer solubility problems, a relatively low buffer concentration of 20 mM was selected.

3.2.4. Gradient condition

According to above results, increasing the methanol content led to a desirable decrease in the FLU retention time. However, the retention time of THZ was also very short (approximately 2 min). The phosphate buffer was sufficient to achieve a good column efficiency (number of theoretical plates) that is necessary for maintaining good peak shapes. Isocratic elution with the phosphate buffer (20 mM, pH 5.0) and methanol (40:60, v/v) mobile phase could not be used due to the different retention properties of THZ and FLU. Therefore, it was necessary to use the gradient elution mode instead of the isocratic elution mode. Trials were carried out, during which, the composition of buffer and methanol at a fixed flow rate of 1.0 mL/min was changed. Among the different trials performed, a gradient program was finalized, in which both components were well separated (*Table* 2).

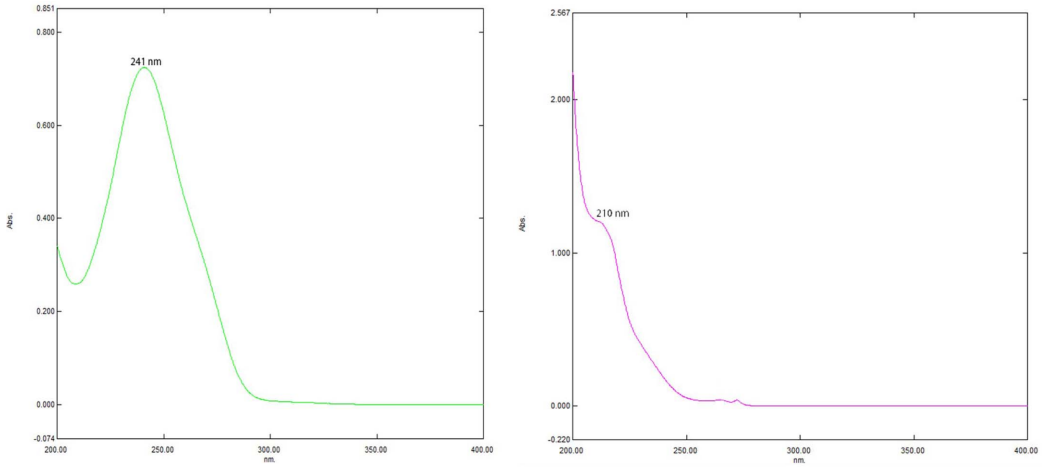
3.2.5. Selection of the detection wavelength

Based on the UV spectra of FLU and THZ, the maximum absorbance wavelengths of FLU and THZ

Table 2. Gradient program

Time (min)	Eluent A (%)	Eluent B (%)
0 – 4	70 → 35	30 → 65
4 – 9	35	65
9 – 10	35 → 70	65 → 30
10 – 15	70	30

Eluent A: 20 mM potassium dihydrogen phosphate at pH 5.0 and methanol (9:1, v/v).
Eluent B: Methanol.



(a) (b)

Fig. 2. UV spectrum of (a) fluorometholone, (b) tetrahydrozoline hydrochloride.

were approximately 241 nm and 210 nm, respectively (*Fig.* 2). Hence, different wavelengths between 210 nm and 260 nm were tested to identify the most suitable detection wavelength. Finally, 220 nm was chosen as the wavelength for quantifying both compounds, due to satisfactory responses and it being an appropriate wavelength for most commercial products of this combination.

3.3. Method validation

3.3.1. Linearity

Calibration curves were linear in the concentration range for FLU between 50 to 600 μg/mL and for THZ between 12.5 to 150 μg/mL (*Table* 3). The coefficient of determination was 0.9999 for FLU and 1.0000 for THZ, which indicates a good correlation between the peak areas and the range of concentrations studied. The LOD and LOQ concentrations of FLU were estimated to be 5.0 and 10.0 μg/mL, respectively while the LOD and LOQ values of THZ and 0.5 and 1.25 μg/mL, respectively, when signal-to- noise ratios of 3 and 10 were used as the criteria (*Table* 3).

3.3.2. Precision

The precision of the method was assessed by determining the intra-day assay relative standard deviation (RSD %) from analysis (n = 6) of the standard solutions at three different concentrations (160, 200, 240 μg/mL of FLU and 40, 50, 60 μg/mL of THZ). Three replicates of each concentration were analyzed over three consecutive days, and the results are shown in *Table* 4. The intra-day precision for each concentration was 0.14-0.30 % and the inter-day precision was 0.16-0.49 % for both FLU and THZ.

3.3.3. Accuracy (recoveries)

For the ophthalmic suspensions, the results of the recovery studies that were carried out with the standard addition method ranged from 99.57 % to 99.72 % for FLU and from 99.84% to 100.17 % for THZ (*Table* 5). This also suggests that there was no interference from excipients in determining the FLU and THZ content in the ophthalmic suspensions.

Table 4. Results of precision (intra/inter-day) validations of the proposed method

Com-pound	Conc. (μg/mL)	Intra-day (n=6)		Inter-day (n=12)	
		RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
FLU	160	0.14	101.48	0.16	100.49
	200	0.20	101.10	0.18	100.70
	240	0.22	99.31	0.18	100.03
THZ	40	0.30	100.56	0.49	100.58
	50	0.30	100.60	0.32	100.05
	60	0.13	101.06	0.29	99.88

Table 5. Recovery tests for FLU and THZ in ophthalmic suspension (n = 3)

Added conc. (%)	FLU		THZ	
	Mean of recovery (%)	RSD (%)	Mean of recovery (%)	RSD (%)
80	99.72	1.03	99.93	0.65
100	99.60	0.49	99.84	0.69
120	99.57	0.73	100.17	1.01

Table 3. Results of linearity validation

Parameter	THZ	FLU
Regression equation	y = 48.196x − 35.433	y = 26.209x
Range (μg/mL)	12.5 − 150	50 − 600
Coefficient of determination (r^2)	1.0000	0.9999
Number of data points	6	6
Slope ± SD	48.196 ± 0.312	26.209 ± 0.211
Intercept ± SD	-35.433 ± 9.489	
Limits of detection (LOD) (μg/mL)	0.5	5.0
Limit of quantitation (LOQ) (μg/mL)	1.25	10.0

SD: Standard deviation

Table 6. System suitability data (n = 6)

Compound	Retention time (RSD %)	Peak area (RSD %)	Theoretical plates number	Asymmetry factor
FLU	0.05	0.07	27128.33	1.39
THZ	0.05	0.20	8730.00	1.12

Table 7. Contents of FLU and THZ in ophthalmic suspension (n = 6)

Compound	Claimed value	Assay	
		Content (%)	RSD (%)
FLU	1 mg/mL	100.72	1.31
THZ	0.25 mg/mL	99.20	0.58

3.4.4. System suitability, robustness, and intermediate precision

The RSD % of the retention time, peak areas and number of theoretical plates, and asymmetry factor were measured after six repeats of 200 µg/mL FLU and 50 µg/mL THZ solution analyses to evaluate the system suitability of the method (*Table* 6). The RSD % of all parameters were less than 2 %. The number of theoretical plates at peak FLU and THZ content were approximately 8700 and 27000, respectively. The asymmetry factors at peak FLU and THZ content were approximately 1.39 and 1.12, respectively.

The robustness of the method was checked by making small and deliberate changes to the pH of phosphate buffer (5.0 ± 0.2) and flow rate (1.0 ± 0.1 mL/min). In both cases, except for slight changes in retention time, the results of the method were not affected *i.e.* the RSD % of the retention time and the peak areas (n = 6) did not exceed 0.3 %. The number of theoretical plates at peak FLU and THZ content were more than 8600 and 26000, respectively. The asymmetry factors at peak FLU and THZ content were less than 1.59 but more than 1.06.

The intermediate precision was studied using a Shimadzu HPLC system. The results show that there was an increase in the retention time of the two

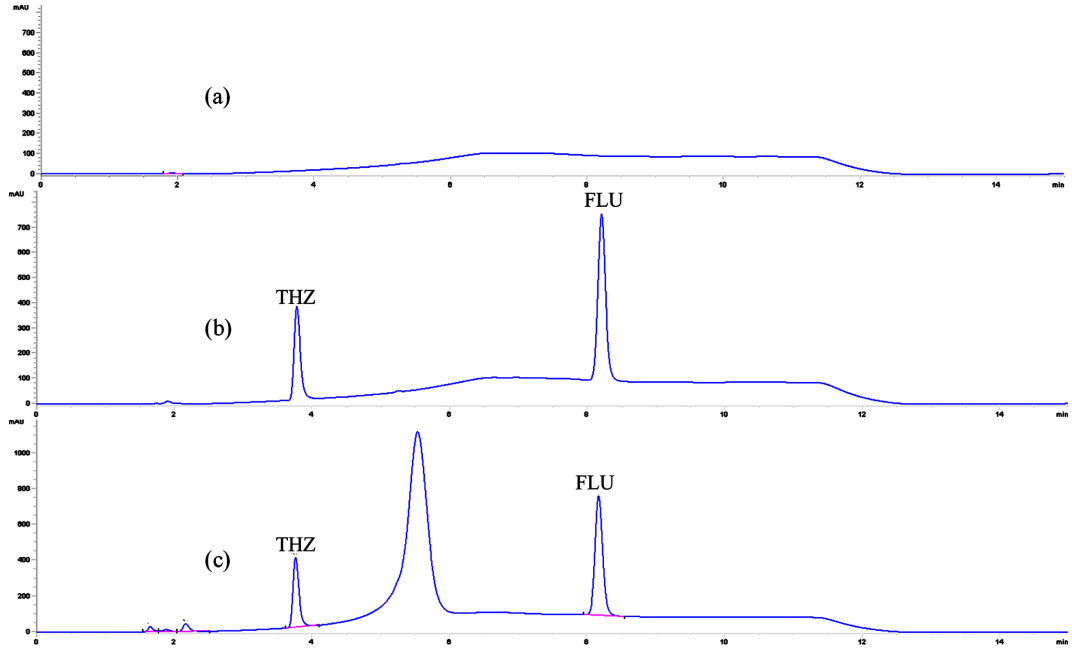


Fig. 3. Typical chromatograms of (a) blank mobile phase sample, (b) 200 µg/mL of FLU and 50 µg/mL of THZ standard solution, (c) sample solution prepared from ophthalmic suspensions. Condition: An Aegispak C18-F column (150 × 4.6 mm I.D., 5 µm) was used for gradient elution by using a binary mixture of eluent A (20mM potassium dihydrogen phosphate at pH 5.0 and methanol (9:1, v/v)) and eluent B (methanol) at a flow rate of 1.0 mL/min, injected volume was 20 µL, UV detection was at 220 nm.

major peaks in comparison to those obtained by the Agilent HPLC system. In the case of peak THZ content, the retention time was approximately 4.2 min, the tailing factor was 1.49, and the number of theoretical plates was greater than 3200. In case of peak FLU content, the retention time was approximately 10.6 min, the tailing factor was 1.13, and the number of theoretical plates was more than 42000. The RSD % (n = 6) of all parameters were lower than 2 %.

3.5. Application

This analytical method was applied for the simultaneous quantification of FLU and THZ content in ophthalmic suspensions. The results of assay tests on six samples of commercial ophthalmic suspensions are recorded in *Table* 7. The average content of FLU in the formulation was 100.72 %, and the RSD % of the samples was 1.31 %. The average THZ content in the formulation was 99.20 %, and the RSD % of the samples was 0.58 %. A typical chromatogram of the samples is shown in *Fig.* 3(c).

4. Conclusions

The proposed study in this paper describes an HPLC method to simultaneously determine THZ and FLU content in ophthalmic suspensions. The method was validated and found to be sensitive, accurate, and precise. Hence, it can be employed in quality control laboratories and the industry for routine quality controls of ophthalmic suspensions that contain both components.

Acknowledgements

This study was supported by a Grant (16172MFDS152) from Ministry of Food and Drug Safety in 2016. The authors thank the Institute of New Drug Development Research and the Central Laboratory of Kangwon National University for the use of their analytical instruments.

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