Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 331–337
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Simultaneous determination of active ingredients in an ophthalmic solution by isocratic tandem-mode HPLC connected reverse phase column and strong cation exchange column

Takahiro Marunouchi *, Masaki Ono, Toshiaki Nakajima, Yuji Ito, Takao Aketo

Analytical Laboratory, Taisho Pharmaceutical Co., Ltd., 403 Yoshino-cho 1-chome, Kita-ku, Saitama-shi, Saitama 331-9530, Japan

Received 5 April 2005; received in revised form 1 August 2005; accepted 4 August 2005
Available online 19 October 2005

Abstract

A tandem-mode high performance liquid chromatography (HPLC) system is described here, which employs reversed phase liquid chromatography (RPLC) followed by strong cation exchange liquid chromatography (SCX), was used to determine the mixture of six ingredients in an ophthalmic solution. As a result of investigations, isocratic HPLC methods that using two columns in tandem-mode; Atlantis dC18 (75 mm × 4.6 mm i.d., 3 μm, ODS) and CAPCELL PAK SCX UG80 (75 mm × 4.6 mm i.d., SCX), which have different separation modes, and control of mixture of methanol/ammonium dihydrogenphosphate buffer as used for the eluent, allowed for six target ingredients to be determined simultaneously. And all ingredients separated perfectly and were determined efficiently and rapidly. Validation of the method was accomplished with respect to linearity ($r > 0.999$), recovery (99.4–100.4%), precision (R.S.D. 0.1–0.9%) and specificity. These results suggest that the fusion of different separation modes can be used for the simultaneous determination of ingredients in ophthalmic solutions, and this can be accomplished rapidly and with high precision.

Keywords: Tandem-mode; Ophthalmic solution; Reversed-phase chromatography; Cation change chromatography

1. Introduction

Commercial ophthalmic solutions contain many ingredients that have different chemical characteristics, thereby making it difficult to determine the ingredients of pharmaceuticals simultaneously with a single method by use of conventional HPLC. However, as regards performing quality control on a commercial basis, of prime importance is the minimization of number of methods used. This means many ingredients have to be determined with one analytical method. The target ingredients for determination were ε-aminocaproic acid (EAC), chlorpheniramine maleate (CP), glycyrrhizic acid dipotassium salt (GK2), neostigmine methyl sulfate (NM), pyridoxine hydrochloride (VB6) and tetrahydrozoline hydrochloride (TH), which are the major six active ingredients in ophthalmic solutions. Generally, EAC, CP, VB6 and TH can be determined by three strong cation exchange (SCX)–HPLC methods, and the others can be determined by two RPLC methods for their chemical properties, thus a long period of time was required to determine one product for quality control. In addition to the above, each ophthalmic solution includes various excipients that sometimes interfered with determination, so the prescription used for the separation method has to be changed. As commonly known, it is impossible to simultaneously determine all ingredients using one ODS or SCX column because of the various characteristics of ingredients. We, therefore, decided to investigate the possibility of combining the characteristics of several columns in order to obtain sufficient separation, even in an isocratic mode. We named this method tandem-mode HPLC (TM-HPLC).

Although a CE method in which the similarity of ingredients is simultaneously determined is currently in operation [1], injection repeatability with CE is generally inferior to HPLC. The method of gradient analysis using HPLC was also considered, however, it is necessary to return the system to the initial state after analysis is completed, thus reducing throughput

* Corresponding author. Tel.: +81 48 669 3047; fax: +81 48 663 1045.
E-mail address: t.marunouchi@po.rd.taisho.co.jp (T. Marunouchi).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.08.018

for multi-sample analysis. Moreover, although there are many reports concerning combinations of different separation modes, column switching is used in almost all cases [2–5]. In the above methods, the first column is used for pretreatment, and after a column switch and eluent change are performed, the second column is used as the main separation column. On the other hand, in the second mode of 2D-HPLC, the ion-exchange column was developed using a step elution, with each step being alternately trapped on two RP columns, which were developed using identical gradient conditions as with the continuous elution system [6]. This is used for the purpose of so-called on-linezing of the re-analysis after fraction collection. Therefore, both columns were not directly linked and the method did not perform isocratic conditioning. Another method, developed by Miyairi et al., has been used to determine the presence of metallothionein with two columns connected in tandem [7]. This method is similar to the one reported in this paper, however, differs in that Miyairi's method utilizes two RPLC columns with a different base particle, and does not combine differing separation modes. Other methods are also in use, such as performing analysis under isocratic conditions by combining an ODS column and polybutadiene-coated zirconia column, whose characteristics are similar to those of cation exchange [8], and performing detection of trace ingredients by filling the gel, which has as different separation mode, into the ionization spray of LC/MS [9]. Other reported methods were; the method in which the gel, which has different separation mode, is filled in one column [10,11], and the method in which the functional group, which has two separation mechanisms, is introduced into silica gel [12]. Although separation of two or more ingredients was attained, there have been no reports on the analysis of pharmaceutical preparations using the mixed mode single column, and moreover, this method is a technique with little flexibility in respect of selection of columns. Moreover, as a best alike method, the method, which connected the chiral column and the SCX column for the chiral separation of two or more ingredients, was reported recently [13].

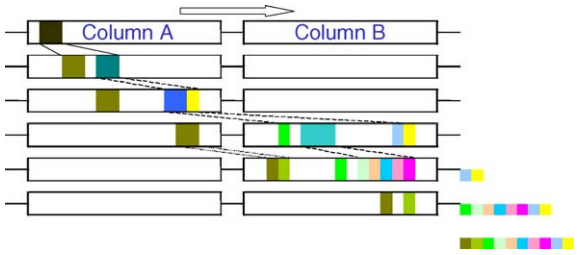

Fig. 1. Model of TM-HPLC method.

In this report, a basic study was carried out for simultaneous determination of ingredients of an ophthalmic solution using HPLC. The object of our investigation was to provide a technique offering flexibility as regards HPLC method, and one that is fast, simple, and uninfluenced by the various excipients that exist in ophthalmic solutions. We formulated a unique method involving two columns (two separation modes) connected in tandem to separate the ingredients in pharmaceutical preparations that were insufficiently separated with one column, and performed analysis with the isocratic mode (Fig. 1). This method was used since it is fast, precise and simple.

2. Experiment

2.1. Reagents

The chemical structures of the active ingredients are shown in Fig. 2. Glycyrrhizic acid dipotassium salt were purchased from Maruzen Pharmaceuticals Co., Ltd. (Hiroshima, Japan), neostigmine methyl sulfate from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), pyridoxine hydrochloride from ROCHE (Basel, Switzerland), tetrahydrozoline hydrochloride from Okami Chemical Industries, Co., Ltd. (Kyoto, Japan), chlorpheniramine maleate from Kongo Chemical Co., Ltd. (Toyama, Japan), ε-aminocaproic acid from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan) and glycyrrhizic acid monoammonium salt from

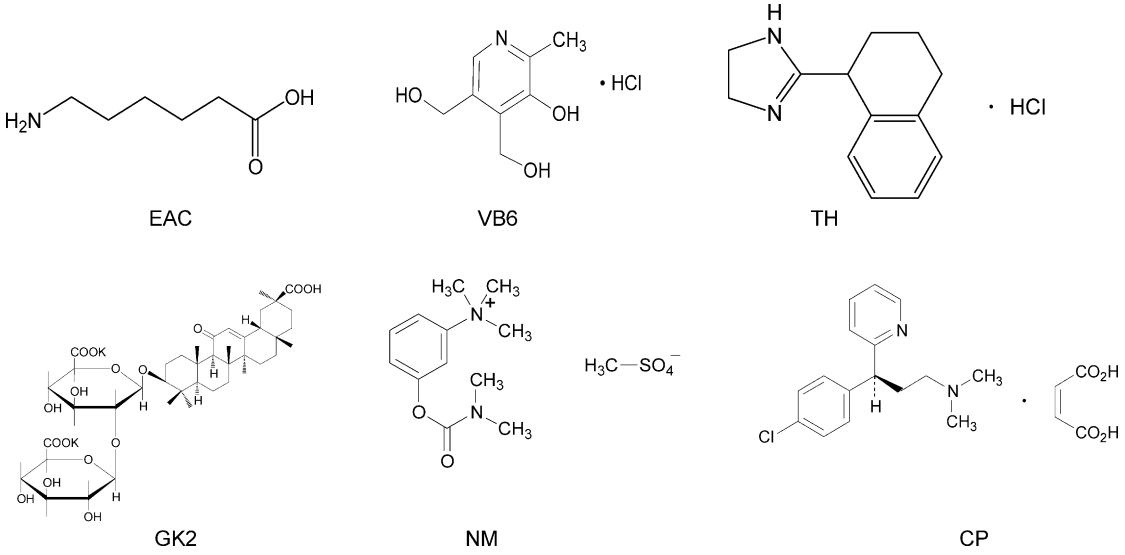

Fig. 2. Structures of six active ingredients.

Alps Pharmaceutical Industries Co., Ltd. (Gifu, Japan), as reference standards.

Allantoin (AL) was purchased from Kawaken Fine Chemicals Co., Ltd. (Tokyo, Japan), 1-chlorobutanol (CB) from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan), benzalkonium chloride (BC) from Kao Corporation (Tokyo, Japan), 4-hyderoxybenzoic acid methyl ester (Me-P) and 4-hydroxybenzoic acid propyl ester (Pr-P) from Midori Kagaku Co., Ltd. (Tokyo, Japan) as excipient regents. 4-Hydroxybenzoic acid 2-ethylhexyl ester was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) as an internal standard (I.S.) for HPLC analysis. Potassium dihydrogenphosphate, ammonium dihydrogenphosphate and phosphoric acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), methanol from Kokusan Chemical Co., Ltd. (Tokyo, Japan) used for the eluent.

Most of the reagents used were of analytical grade, except for those requiring higher specifications.

2.2. Instrumentation

The HPLC systems: Waters 2695 equipped with photo diode array (PDA) Detector (Waters 2996), were purchased from Waters (Milford, USA).

2.3. Columns

CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d. and 75 mm × 4.6 mm i.d., 5 μm) SCX columns were purchased from Shiseido (Tokyo, Japan).

Atlantis columns (150 mm × 4.6 mm i.d. or 75 mm × 4.6 mm i.d., 3 μm) were purchased from Waters, Chromolith Performance (100 mm × 4.6 mm i.d.) and Chromolith SpeedROD (50 mm × 4.6 mm i.d.) columns were purchased from Merck (Darmstadt, Germany), and CAPCELL PAK C18 AQ (150 mm × 4.6 mm i.d., 5 μm) from Shiseido and Hydrosphere C18 ODS columns (150 mm × 4.6 mm i.d., 5 μm) were purchased from YMC (Kyoto, Japan).

2.4. Standard and sample solutions

2.4.1. Target ingredients

The following six ingredients used widely in ophthalmic solutions were used as target ingredients.

Target ingredients: GK2, VB6, EAC, NM, TH and CP.

2.4.2. Preparation of the solutions

In a 10 ml volumetric flask was placed 2.0 ml of a pharmaceutical preparation, then added with 2.0 ml of internal standard solution, diluted with water–methanol (1:1, v/v) to volume, and mixed to obtain a sample solution. For the preparation of the standard solution, the six ingredients were weighed and diluted with water–methanol (1:1, v/v) so that the concentration becomes similar to that of the prepared sample solution. The volume of the internal solution that was added to the standard solution was identical to that of the standard solution. The internal standard solution was prepared by dissolving 10 mg of 4-hydroxybenzoic acid 2-ethylhexyl ester in 500 ml of methanol.

2.5. Theoretical formula used to determine retention time in tandem-mode HPLC analysis

Using the following formula (1), we predicted that retention time with two columns connected in tandem (t_{tandem}) is obtained from the retention time of the ODS column (t_{ODS}) and SCX column (t_{RSCX}):

$$t_{Rtandem} = t_{RODS} + t_{RSCX} - t_{Rblank} \quad (1)$$

t_{Rblank} = 0.10 min. The time required for a sample to pass through equipment when there are no columns.

3. Result and discussion

3.1. Optimization of analytical conditions

The eluent was made from a buffer solution–organic solvent mixture because of using both ODS and SCX columns together. The buffer solution contained potassium dihydrogenphosphate or ammonium dihydrogenphosphate. Methanol was used for the organic solvent.

The salt concentration of the buffer solution was maintained within the range of about 50–150 mM.

In conformity with JP14, complete separation of the peak means that the resolution between two peaks is not less than 1.5.

3.1.1. Eluent A: Eluent using potassium dihydrogenphosphate as the salt

3.1.1.1. Separation examination of target ingredients and antiseptics, and verification of a formula (1). Since it was necessary to also separate antiseptics with UV absorption with target ingredients, separation of nine ingredients containing six target ingredients and three antiseptic ingredients (Me-P, Pr-P and CB) was considered. Preliminary examination showed that it is possible, by inserting the actual retention time of each ingredient in the formula (1), to predict the retention time when connecting two columns (Table 1). Moreover, it was also found that the retention time of each ingredient basically unaffected by the connection order of a column. Formula (1) was used to determine sufficient separation, and then the two columns were connected to determine precise separation. From Table 1, it can be seen that no ingredient was retained completely in either column, and GK2, CB and Pr-P were retained more in the ODS column than the SCX column, and that other basic ingredients were retained more in the SCX column than the ODS column.

The typical chromatogram formed by eluent A is shown in Fig. 3. The method shown in Fig. 3 could determine not only target ingredients but also other ingredients, such as a preservative agents, thus it has a wide range of applications. However, peaks eluted in the crowded domain for less than 10 min with poor separation were very sensitive to slight change in composition of eluent, degradation of column, etc., therefore, it was considered that improvement of separation of this domain was

Table 1
Theoretical retention time and actual retention time

t_R	Actual measurement (min)			Prediction value (min)	Prediction—actual (min)
	t_{RODS}	t_{RSCX}	$t_{RTandem}$	$t_{RODS} + t_{RSCX} - t_{blank}$	
t_0	0.468	1.460	2.300	1.828	−0.47
EAC	1.001	4.544	5.351	5.445	0.09
VB6	1.008	4.986	5.836	5.894	0.06
NM	1.084	6.306	7.223	7.290	0.07
TH	1.419	9.014	10.306	10.333	0.03
CP	2.573	22.416	25.683	24.889	−0.79
GK2	53.767	1.932	50.027	55.599	5.57
Me-P	2.338	2.308	4.424	4.546	0.12
Pr-P	6.144	2.477	8.236	8.521	0.29
CB	4.534	2.305	6.569	6.739	0.17

ODS: Atlantis (75 mm × 4.6 mm i.d., 3 μm particle size; Waters) SCX: CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d., 5 μm particle size; Shiseido). Eluent: methanol–aqueous solution made up using phosphoric acid added to potassium dihydrogenphosphate (150 mM), adjusted to pH 3.0 (53:47, v/v).

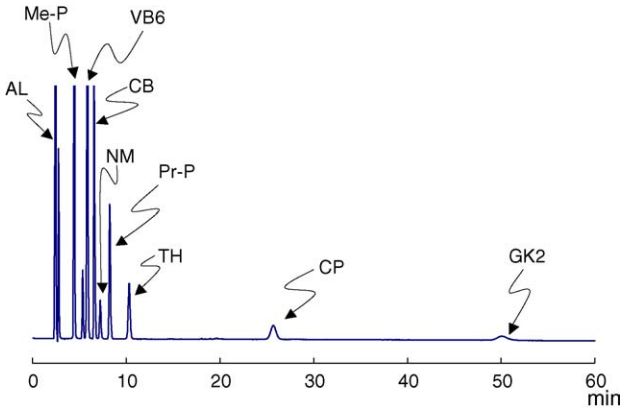

Fig. 3. A typical chromatogram of TM-HPLC (eluent A). The detector was set at 210 nm. Separation was carried out at 50 °C using Atlantis (75 mm × 4.6 mm i.d., 3 μm particle size; Waters, ODS) and CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d., 5 μm particle size; Shiseido, SCX) connected in tandem. The mobile phase consisted of methanol–aqueous solution which added phosphoric acid to potassium dihydrogenphosphate (150 mM), and was adjusted to pH 3.0 (53:47, v/v), at a flow rate of 1.0 ml min^{-1}.

required. Moreover, GK2 and BC eluted to the late domain of retention time (more than 60 min). Compared to other ingredients, these ingredients tend to be sensitively influenced by any change in composition of eluent. Therefore, it was necessary to perform the gradient method to remove the late-eluted ingredients of a column, or isocratic method, in which complete elution is waited for. It was concluded that the latter method was not suitable for simultaneous determination of multiple samples due to the excessive time needed for analysis, and high consumption of eluent. There was plenty of scope for improvement to make GK2 and BC elute at an earlier time, and to suppress changes in retention time as much as possible.

3.1.1.2. Effect of methanol proportions. During method development, the effects of eluent composition on analyte retention were evaluated, and these are represented in Fig. 4 as analyte retention time versus methanol proportion. Increasing the proportion of methanol in the eluent decreased the retention time of all the analytes. It was observed that the retention time of the ingredient strongly retained in an ODS column varied greatly.

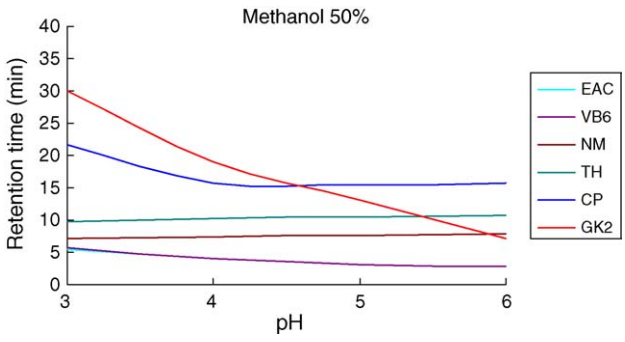

Fig. 4. The effect of pH in eluent on retention time.

3.1.1.3. Effect of pH of buffers. Further, the effects of eluent composition on analyte retention were evaluated and a graph of analyte retention time versus pH is presented in Fig. 5. The pH of the eluent was varied between 3 and 6. The retention time of GK2 changed the most, and there was almost no change in other ingredients. The ion strength of each eluent differed because phosphoric acid was added without adjusting its concentration when adjusting the pH of buffers. It is thought that most ingredients did not change their retention because influence of ion strength balanced with influence of pH. From this result, it was judged that almost all the ingredients were relatively uninfluenced, thus the pH of the buffer was fixed at 3.0, in consideration of pK_a of EAC and VB6.

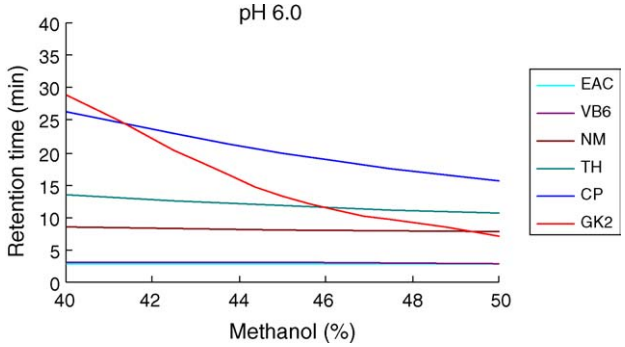

Fig. 5. The effect of methanol ratio in eluent.

Table 2
Preferable combinations of columns for separation and methanol (%) of eluent A

No.	ODS column	SCX column	Methanol (%)
1	Atlantis (75 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d.)	47
2	Hydrosphere C18 (150 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d.)	44
3	Chromolith Performance (100 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d.)	44
4	CAPCELL PAK C18 AQ (150 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (75 mm × 4.6 mm i.d.)	50
5	Chromolith Performance (100 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (75 mm × 4.6 mm i.d.)	46

3.1.1.4. Effect of column connection. As a result of simulations using formula (1) and following actual analysis, the best combination of columns and methanol percentage of eluent was found from the point at which all ingredients separated sufficiently, as shown in Table 2.

3.1.2. Eluent B: Eluent using ammonium dihydrogenphosphate as the salt

3.1.2.1. Effect of the concentration of buffer. A buffer made from ammonium dihydrogenphosphate and phosphoric acid was mixed with methanol at an arbitrary rate with no precipitation of salt. Therefore, the HPLC conditions that used ammonium dihydrogenphosphate for the eluent were examined. Eluent B-1, a methanol–aqueous solution made up with phosphoric acid added to ammonium dihydrogenphosphate (50 mM) and adjusted to pH 3.0, and eluent B-2, a methanol–aqueous solution made up with phosphoric acid added to ammonium dihydrogenphosphate (150 mM) and adjusted to pH 3.0 were used. Also in this examination, the optimal separation condition was calculated using formula (1).

Since most analytical ingredients mainly interacted in the SCX column, separation conditions of the ingredients that interacted in the SCX column were examined for a methanol content of from 10 to 80% using solely the SCX column. Based on the examination result of Section 3.1.1.3, the pH of the eluent was fixed at 3.0. Almost all the ingredients were roughly separated at a methanol content of 60–70% in the case of the 50 mM ammonium dihydrogenphosphate buffer, and also roughly separated at 40–50% in the case of the 150 mM ammonium dihydrogenphosphate buffer. Then, the retention time and separation when connecting two columns was predicted after the observation of actual retention time using the ODS column.

The result of separation using two connected columns, the preferable combinations of columns, and the methanol percentage in the eluent that enabled all ingredients to separate sufficiently are shown in Tables 3 and 4.

When performing analysis using two connected columns, the chromatogram patterns were classified into two types. One of the patterns was obtained using eluent A. In this pattern, determination of excipients was possible, as in the case of using potassium dihydrogenphosphate as the eluent, and many peaks formed in a short period of time. Another pattern was shown Fig. 6. In this pattern, although most excipients were eluted almost to t_0 and could not be determined, the target ingredients were sufficiently separated.

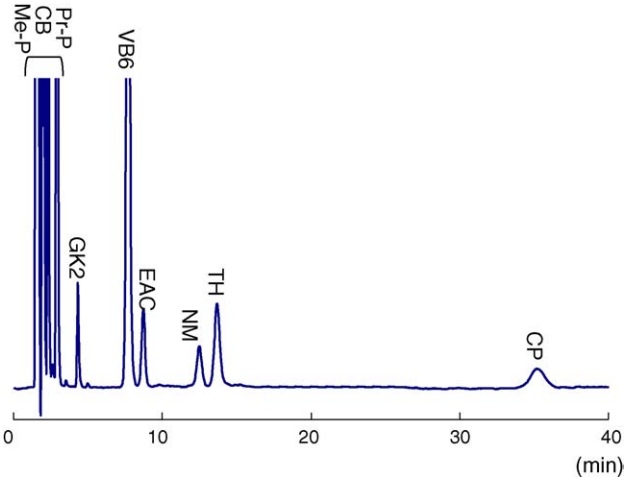

Fig. 6. A typical chromatogram of TM-HPLC (eluent B-1). The detector was set at 210 nm. Separation was carried out at 50 °C using Atlantis (75 mm × 4.6 mm i.d., 3 μm particle size; Waters, ODS) and CAPCELL PAK SCX UG80 (75 mm × 4.6 mm i.d., 5 μm particle size; Shiseido, SCX) connected in tandem. The mobile phase consisted of methanol–aqueous solution which added phosphoric acid to ammonium dihydrogenphosphate (50 mM), and was adjusted to pH 3.0 (63:37, v/v), at a flow rate of 1.0 ml min^{-1}.

The pattern shown in Fig. 6 was obtained with an Atlantis (75 mm × 4.6 mm i.d., 3 μm; Waters, ODS) connected to a CAPCELL PAK SCX UG80 (75 mm × 4.6 mm i.d., 5 μm; Shiseido, SCX), the eluent being a methanol–aqueous solution made up by mixing phosphoric acid and ammonium dihydrogenphosphate (50 mM) and adjusted to pH 3.0 (67:33, v/v). And in other conditions, the pattern shown in Fig. 5 was obtained. The chromatograph pattern as shown in Fig. 6 was obtained when separation of the ingredients that were mainly retained in the SCX column was completed, and retention of the ingredients that mainly retained in the ODS column was comparatively weak.

Table 3
Preferable combinations of columns for separation and methanol (%) of eluent B-1

No.	ODS column	SCX column	Methanol (%)
1	Atlantis (150 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d.)	67
2	Hydrosphere C18 (150 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d.)	65–69
3	Atlantis (75 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (75 mm × 4.6 mm i.d.)	65–67
4	Hydrosphere C18 (150 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (75 mm × 4.6 mm i.d.)	64

Table 4
Preferable combinations of columns for separation and methanol (%) of eluent B-2

No.	ODS column	SCX column	Methanol (%)
1	Atlantis (150 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d.)	49–50
2	Atlantis (75 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d.)	41–43
3	CAPCELL PAK C18 AQ (150 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d.)	47–49
4	Chromolith SpeedROD (50 mm × 4.6 mm i.d.)	CAPCELL PAK SCX UG80 (150 mm × 4.6 mm i.d.)	45–47

In the pattern of Fig. 6, GK2 eluted at a relatively early retention time. Moreover, it was also notable that BC eluted within 40 min of analytical time, therefore, this method was used for the basic analytical conditions in this study, and for analysis method validation.

As stated above, the SCX column mainly separated the most basic ingredients. In the case of connecting two columns in a SCX–ODS order, when a high-concentration sample solution was injected, the peak was split especially at the peak of VB6 due to overload of the SCX column. Reducing the concentration of the sample solution easily rectified this undesirable characteristic. However, this brought about a negative influence on determination accuracy, since the peaks of ingredients with low concentrations become too small. It was stated above that the connection order of a column rarely affects separation of each ingredient. When connecting the column in an order contrary to the former, the sample introduced into a SCX column could be distributed because a sample previously passes through the ODS column, and therefore, an improvement of peak form would be expected An improvement in peak form was demonstrated from the results of experiments. As compared with the technique using the previously reported methods [9–11], a larger amount of samples could be injected in the present method owing to the use of "pre-separation" columns.

3.2. Validation of analytical procedures

Validation of analytical procedures was carried out according to the following established HPLC conditions. In addition, since GK2 eluted to the early domain of retention time and was easily affected by the presence of other ingredients, it was detected at 254 nm, since detection sensitivity is higher above 200 nm.

The established HPLC condition was described here. The detector was set at 210 nm for VB6, EAC, NM, TH and CP and 254 nm for GK2. Separation was carried out at 50 °C using Atlantis (75 mm × 4.6 mm i.d., 3 μm particle size; Waters, ODS) and CAPCELL PAK SCX UG80 (75 mm × 4.6 mm i.d., 5 μm particle size; Shiseido, SCX) connected in tandem. The mobile phase consisted of methanol–aqueous solution comprising phosphoric acid added to ammonium dihydrogenphosphate (50 mM), adjusted to pH 3.0 (67:33, v/v), at a flow rate of 1.0 ml min^{-1}. All calculations concerning the quantitative analysis were performed with internal standardization by measurement of peak areas.

Table 6
Validation data for determination of ingredients in ophthalmic solution (recovery, precision)

	VB6	EAC	NM	TH	CP	GK2
Recovery (n = 3, %)						
Concentration range (%)						
80	100.1	100.3	100.2	100.2	99.4	100.7
100	100.5	100.5	99.4	100.3	99.7	100.9
120	100.4	100.3	99.9	100.5	99.7	100.9
Precision (n = 3, R.S.D.%)						
Concentration range (%)						
80	0.7	0.4	0.7	0.7	0.6	0.3
100	0.6	0.3	0.6	0.3	0.9	0.2
120	0.4	0.3	0.4	0.3	0.7	0.2

All ingredients showed favorable results in this concentration range.

3.2.1. Linearity

The linearity in the over/under ranges from less than 50% to more than 150% of the normal concentrations was checked, the results of which are given in Table 5.

3.2.2. Recovery and precision

A recovery and precision test in the range of 80–120% of the normal concentration was performed. Sample solutions were made from a placebo and equal volumes of standard solution. The results are shown in Table 6.

3.2.3. Specificity

We prepared a sample of ophthalmic solution and checked singularity.

Determination was carried successfully.

Table 5
Validation data for determination of ingredients in ophthalmic solution (linearity)

	VB6	EAC	NM	TH	CP	GK2
Linearity						
Concentration range (μg/ml)	50–150	500–1500	0.5–7.5	12.5–75	7.5–45	12.5–375
R	0.9999	0.9994	0.9994	0.9999	0.9996	0.9999
Intercept	−0.3525	−0.0497	−0.0117	−0.0447	−0.0941	+0.0007
Slope	0.2273	0.001244	0.0950	0.1927	0.1208	0.03270

All ingredients were confirmed to have favorable linearity in this concentration range.

Table 7
Difference between column batches

Column set no.	Column	Column Lot.	Gel batch	Methanol ratio upon sufficient separation (%)
1	Atlantis	T20671K09	101	66.0
	CAPCELL PAK SCX UG80	HQAI01015	H-10	
2	Atlantis	W2316V02	103	66.5
	CAPCELL PAK SCX UG80	HQAI01007	H-11	
3	Atlantis	W32811R03	109	67.5
	CAPCELL PAK SCX UG80	HQAI01013	H-12	

3.2.4. Difference between gel batches

The combinations of columns used for this examination and the methanol content in the eluent when sufficient separation of all the ingredients was obtained for determination is shown in Table 7.

Sufficient separation was obtained in the same order of elution by changing the methanol content in eluent approximately ±1% when analyzing three sets of columns, whose gel batches were different from each other. Therefore, it was determined that the difference between gel batches of columns represented no problems. However, depending on the column lot, the elution order of EAC and VB6 may be reversed when methanol content was changed, thus some care is required. The retention time of EAC was easily influenced by methanol content. On the other hand, VB6 was not influenced by methanol content. When the methanol content of the eluent was increased, the retention time of EAC significantly deviated from VB6. It is thus necessary just to adjust the retention time and separation of both ingredients based on the above result.

Table 8
Comparison of TM-HPLC and conventional method

Ingredient	Lot.	TM-HPLC, average ($n = 3$) (R.S.D.%)	Conventional method, average ($n = 3$) (R.S.D.%)
EAC, 1.0%	A	102.1 (0.5)	100.7 (0.6)
	B	101.9 (0.2)	100.7 (0.4)
	C	102.4 (1.2)	100.8 (0.9)
VB6, 0.10%	A	100.4 (0.2)	100.8 (0.1)
	B	99.8 (0.9)	101.2 (0.4)
	C	100.2 (1.0)	101.1 (0.5)
CP, 0.02%	A	99.9 (0.5)	101.0 (0.5)
	B	100.4 (0.8)	101.2 (0.7)
	C	99.9 (0.9)	101.0 (1.0)
NM, 0.002%	A	102.9 (0.4)	100.8 (1.0)
	B	101.7 (1.0)	100.7 (0.6)
	C	101.3 (0.6)	101.1 (1.0)
TH, 0.03%	A	102.3 (0.3)	101.6 (0.3)
	B	102.9 (0.2)	101.8 (0.3)
	C	102.4 (0.8)	102.0 (0.3)
GK2, 0.25%	A	98.3 (0.9)	98.8 (0.3)

3.2.5. Comparison with the conventional method

The conventional method and TM-HPLC method were compared quantitatively using commercial pharmaceuticals.

The results are shown in Table 8. Good agreement was obtained between the conventional method and the TM-HPLC method

4. Conclusion

The TM-HPLC method has been set up as a determination method of six ingredients used widely in ophthalmic solutions as a result of this examination. By having set up this method, the number of analytical conditions for the various ophthalmic solutions was able to be reduced. Therefore, this method enables a rapid setup of the determination method and simultaneous determination, resulting in an increase in efficiency. Furthermore, since an ophthalmic solution consists of many ingredients, this method may also be applied to the determination of the products of other preparations.

References

[1] H. Okamoto, A. Uetake, R. Tamaya, T. Nakajima, K. Sagara, Y. Ito, J. Chromatogr. A 888 (2000) 299–308.
[2] G.J. Opiteck, K.C. Lewis, J.W. Jorgenson, Anal. Chem. 69 (1997) 1518–1524.
[3] G.J. Opiteck, J.W. Jorgenson, Anal. Chem. 69 (1997) 2283–2291.
[4] M.T. Davis, J. Beierle, E.T. Bures, M.D. McGinley, J. Mort, J.H. Robinson, C.S. Spahr, W. Yu, R. Luethy, S.D. Patterson, J. Chromatogr. B 752 (2001) 281–291.
[5] M.M. Bushey, J.W. Jorgenson, Anal. Chem. 62 (1990) 161–167.
[6] N. Tanaka, H. Kimura, D. Tokuda, K. Hosoya, T. Ikegami, N. Ishizuka, H. Minakuchi, K. Nakanishi, Y. Shintani, M. Furuno, K. Cabrera, Anal. Chem. 76 (2004) 1273–1281.
[7] S. Miyairi, S. Shibata, A. Naganuma, Anal. Biochem. 258 (1998) 168–175.
[8] Y. Mao, P.W. Carr, Anal. Chem. 73 (2001) 4478–4485.
[9] D.A. Wolters, M.P. Washburn, J.R. Yates III, Anal. Chem. 73 (2001) 5683–5690.
[10] Y. Bergqvist, C. Hopstadius, J. Chromatogr. B 741 (2000) 189–193.
[11] M. Walshe, M.T. Kelly, M.R. Smyth, H. Ritchie, J. Chromatogr. A 708 (1995) 31–40.
[12] M.M. Muenter, C.C. Stokes, R.T. Obie, J.R. Jezorek, J. Chromatogr. A 844 (1999) 39–51.
[13] I. D'Acquarica, F. Gasparrini, B. Giannoli, E. Badaloni, B. Galletti, F. Giorgi, M.O. Tinti, A. Vigevani, J. Chromatogr. A 1061 (2004) 167–173.