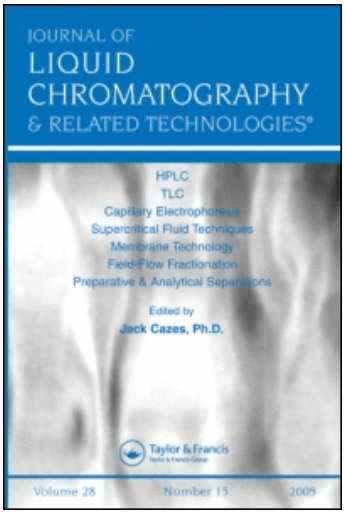

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Development and Validation of a HPLC Method for Determination of Potential Residual Cortisone Compounds in Timolol Maleate Eye Drops

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Abstract: One way of ensuring effective cleaning in the pharmaceutical industry is to analyze the batch of a product for a potential carry over contamination from a previously manufactured one. The potential cross contamination of timolol maleate (TM) eye drops with cortisone compounds, deserves special consideration, due to the well known deleterious effect of cortisones on glaucoma. Therefore, it was the objective of this study to develop and validate a HPLC method for the detection and quantitative determination of cortisones in TM eye drop preparations. The chromatographic behaviors of the five cortisones that were most likely to be present as contaminants in TM eye drops were characterized on different HPLC stationary phases (normal silica, C_{18}, C_8, and CN). Mobile phase and buffer constituents were further optimized on the best stationary phase material (C_{18}). The final recommended mobile phase consisted of a mixture of THF:methanol:0.01M phosphate buffet at ratios of 15:25:60 and containing 0.01 M camphore sulfonic acid (pH adjusted to 4.2). The method was validated in light of ICH guidelines and applied to commercial samples with satisfactory results.

Keywords: Cleaning validation, Cortisones, Eye drops, Timolol maleate

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INTRODUCTION

Timolol maleate (TM, Figure 1) is a beta adrenergic blocker that has been in clinical use for more than three decades.[1] TM is available in 0.25% and 0.5% eye drop solution (Timoptic®) for treatment of open angle glaucoma.[2] Cortisones like prednisolone (PRD), dexamethasone sodium phosphate (DXSP), betamethasone sodium phosphate (BTSP), hydrocortisone acetate (HCA), and flourometholone (FRM), structures shown in Figure 1, are usually used in eye drop preparation for the treatment of various allergic conditions of the eyes.[3]

Cortisones, however, are well known for their deleterious effect on glaucoma as they result in an increase of intraocular pressure.[4–5] It is quite likely that drug manufacturers would use the same filling machine for filling eye drop solutions of various kinds including cortisone based ones. Therefore, it might be possible to have eye drop batches of TM cross contaminated with cortisone compounds from previous batches

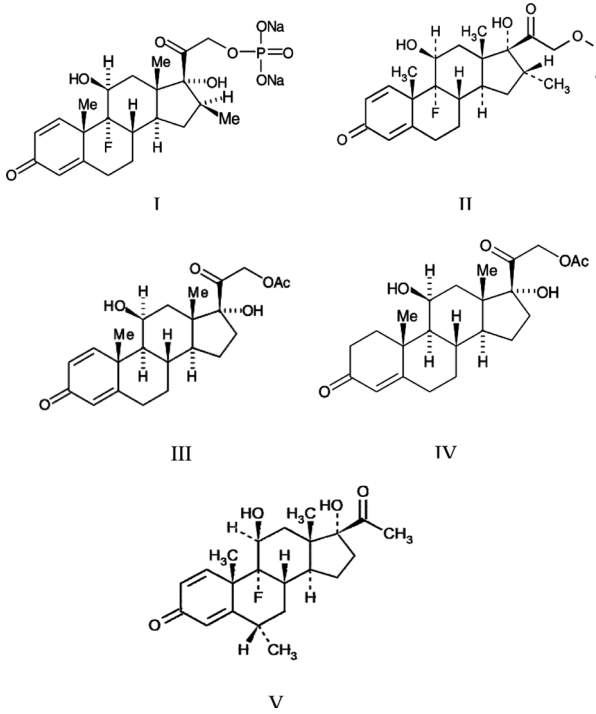

Figure 1. Chemical structure of the examined cortisone compounds. (I) betamethasone sodium phosphate, (II) dexamethasone sodium phosphate, (III) prednisolone acetate, (IV) hydrocortisone acetate and (V) flourometholone.

that have been filled using the same filling machine. Due to the well established deleterious effect of cortisone compounds on intraocular pressure and the fact that a glaucoma patient would have to use TM for long terms, the potential contamination of TM preparation with cortisone compounds is of special concern.

It is the responsibility of the manufacturer to establish an effective and validated cleaning system that ensures no cross contamination would occur between successive batches. General requirements for compliance with cleaning validation regulations were issued by the FDA (see the relevant web site at http://apic.cefic.org/pub/pub-cleaning-validation.pdf). Several techniques have been reported for validating a cleaning system. Such techniques include the swab sampling method, the rinse sampling method, the placebo sampling method, and the product sampling method.[6] The last method (product sampling method) states that the product that is likely to be cross contaminated with a drug from a previous product could be analyzed for residual contaminant.[6] This approach was of particular interest in our laboratory, as it provides a means to control the quality of TM eye drops by both the drug manufacturers, as well as regulatory agencies.[6]

In spite of many analytical methods that have been reported for the analysis of various cortisone compounds none has included the particular five cortisone compounds, which are likely to be present as a carry over from previously manufactured batches of cortisone compounds. These cortisones include DXSP, BTSP, PRD, HCA, and FRM. Moreover, none of the reported methods has dealt with the specific problem of separation, identification, and quantification of cortisones in TM eye drop preparations. Therefore, the aim of this study was to develop a HPLC method that enables the separation, identification, and quantification of the above mentioned cortisone compounds in a TM eye drop preparation. This method would be helpful for drug manufacturers as a part of a cleaning validation system, as well as to laboratories of medicine control agencies that usually test products after being marketed.

EXPERIMENTAL

Chemicals

Working standards of prednisolone acetate, hydrocortisone acetate, timolol maleate, dexamethasone sodium phosphate, and betamethasone sodium phosphate were provided by Amman Pharmaceutical Industries (API, Jordan). Fluoromthalone was from Sigma Aldrich (St. Louis, USA). HPLC grade acetonitrile, methanol, ethanol, and water were purchased from Scharlau (Spain). Tetrahydrofuran was from Tedia

Company (USA). Dichloromethane and glacial acetic acid were purchased from Gainland Chemical Company (GCC, UK). (−)-Camphor –10-sulphonyl chloride was purchased from Fluka Chemie GmbH (Germany) and heptane sulfonic acid sodium salt (HPLC grade) was purchased from Across (Germany). Hydroxy propyl β-cyclodextrin was kindly provided by Jordan Pharmaceutical Manufacturer (JPM, Jordan).

Commercially available eye drops, Apimol® (Timolol maleate, 0.25%), batch No. EA051, Apimol® (Timolol maleate, 0.5%), batch No. EB099; Ophtamolol® (Timolol maleate, 0.25%), Batch No 1496; Ophtamolol® (Timolol maleate 0.5%), batch No. 1419) Timolol® (Timolol maleate 0.5%), batch No. 050997, and Nylol® gel (Timolol 1 mg/mL batch No. 411708) were obtained from the local market.

Equipment

Shimadzu Class-VP HPLC instrument equipped with a SPD-10AV UV/VIS UV detector, LC-2010 integrator, DGU-14A degasser, SIL-10 AD auto injector, CTO-10AS column oven, and LC-10AD pump were employed. Columns used were Waters symmetry C_{18} (150 × 4.6 mm, 5 μm), Varian microsorb C_8, (150 × 4.6 mm, 5 μm), Luna CN (150 × 4.6 mm, 5 μm), and Shimadzu silica (150 × 4.6 mm, 5 μm).

Chromatographic Conditions

Several chromatographic conditions were examined in order to characterize the chromatographic behavior of the cortisone compounds under testing. The finally optimized conditions were: A reversed phase C_{18} column with a mobile phase composed of THF:methanol:0.1 M phosphoric acid at v/v ratios of 15:25:60 containing 0.01 M of camphor sulfonic acid, and the apparent pH adjusted to 4.2 using 5 M NaOH. The mobile phase was prepared daily, filtered through 0.45 μm membrane filters, and sonicated before use. Flow rate was set at 1 mL/min through all experiments. All chromatograms were monitored at 240 nm and, in the case of timolol, detector signals were also monitored at 280 nm for the purpose of confirming identity.

Preparation of Stock Solutions and Standard Mixture

Stock solutions (0.5 mg/mL) of DXSP sodium phosphate, BTSP sodium phosphate, PD acetate, HD acetate, and flourometholone were prepared in methanol. Aliquots from the stock solution of each compound

were diluted with the mobile phase to obtain nine solutions having concentrations in the range 0.04–100 μg/mL.

Standard mixtures containing each of the cortisone compounds in addition to timolol maleate were prepared to evaluate each of the examined conditions. Of the stock solution, 1 mL of each of the cortisone compounds was transferred to a 100 mL volumetric flask, and diluted with the proper mobile phase to obtain final concentration of each compound = 4.8 μg/mL.

Validation and Application of the Proposed Chromatographic Method

Linearity and Precision

Stock solutions (0.5 mg/mL) of each compound were diluted properly to obtain a series of nine standard solutions in the range 0.04–100 μg/mL. Five injections were made at each concentration level and the average value was reported. Calibration curves were constructed for each compound in the specified concentration range. The linearity was assessed using the least square method.

Selectivity

Several additives that are likely to be present in an eye drop preparation were chromatographed using the same conditions of the proposed method. A solution of each substance was prepared in the mobile phase at a concentration of 0.1 mg/mL, which is generally higher than expected levels. The list included: benzalkonium chloride, NaH_2PO_4 dihydrate, boric acid, thiomersal, trometamol (merthiolate), disodium edetate, sodium chloride, sodium meta bisulphate, sodium citrate, hydroxyl propylmethyl cellulose, chlorobutanol, and tromethamine.

Timolol maleate was subjected to enforced degradation by refluxing a saturated solution with HCl (1 M), NaOH (1 M), and (10%) H_2O_2 for 4 hours. The obtained solutions containing the assumed degradation products, were neutralized and then injected into the chromatographic system using the proposed conditions.

Ruggedness

The effect of little changes in pH of the mobile phase (4.2 ± 0.2), temperature of the column (25 ± 2), percentage of methanol (25 ± 2), and percentage of THF (15 ± 2) in the mobile phase was examined. RSD values were obtained for the retention times of each compound when chromatographed at each of the sub-optimum conditions examined.

Application of the Proposed Method on Real Samples

The proposed method was applied in eye drops containing timolol maleate available in the local market (see above section). Of the eye drops solution to be tested, 20 μL were directly injected onto the HPLC column and chromatographed using the proposed method.

RESULTS AND DISCUSSION

The method developed in this study is intended to be used for detection, identification, and quantification of residual cortisones in TM eye drops. Therefore, the method should be able to separate the major cortisone compounds that are most often used in eye drop preparations from each other and from TM. In order to achieve the optimum separation of the test cortisone compounds, systematic characterization of their chromatographic behavior was carried out.

Method development started with determining the retention times of the test compounds on bonded phase columns (C_8, C_{18}, CN) using various ratios of methanol in water (90%, 75%, 55%, and 35%). On the three columns tested, the retention of all cortisone compounds increased when the percentage of methanol was decreased (Figure 2). At the percentage of 35%, the retention of all compounds appeared to be longer than the run time, which was set at 30 min. None of the examined conditions (columns or stationary phases) resulted in a satisfactory separation of the cortisone compounds. It was noteworthy however, that the cortisone compounds could be classified (in all examined conditions) into two groups: the early eluting cortisones (DXSP and BTSP) and the late eluting cortisones (HCA, PRD, FRM). This behavior could be explained as the early eluting cortisone compounds were in the salt form (sodium phosphate) unlike the late eluting ones. The readily ionizable salt form is more polar and consequently elutes faster than the non ionized forms on reversed phase columns.

However a C_{18} column with a mobile phase consisting of 75% methanol was chosen to continue method development, as this condition provided reasonable retention times for all analytes. Therefore, the next attempt was to examine a mobile phase consisting of acetonitrile (ACN) water but with similar eluotropic strength, i.e., isoeluotropic.[7] However, the isoleluotropic strength of ACN (60%) showed no selectivity differences over that produced by methanol (i.e., all cortisone compounds eluted in the same order observed with methanol, see Figure 3).

The effect of pH on resolution of the test compounds was studied by adjusting the pH of the mobile phase in the range 3–6.5. The pH did not seem to have a selective effect on the retention of the cortisone

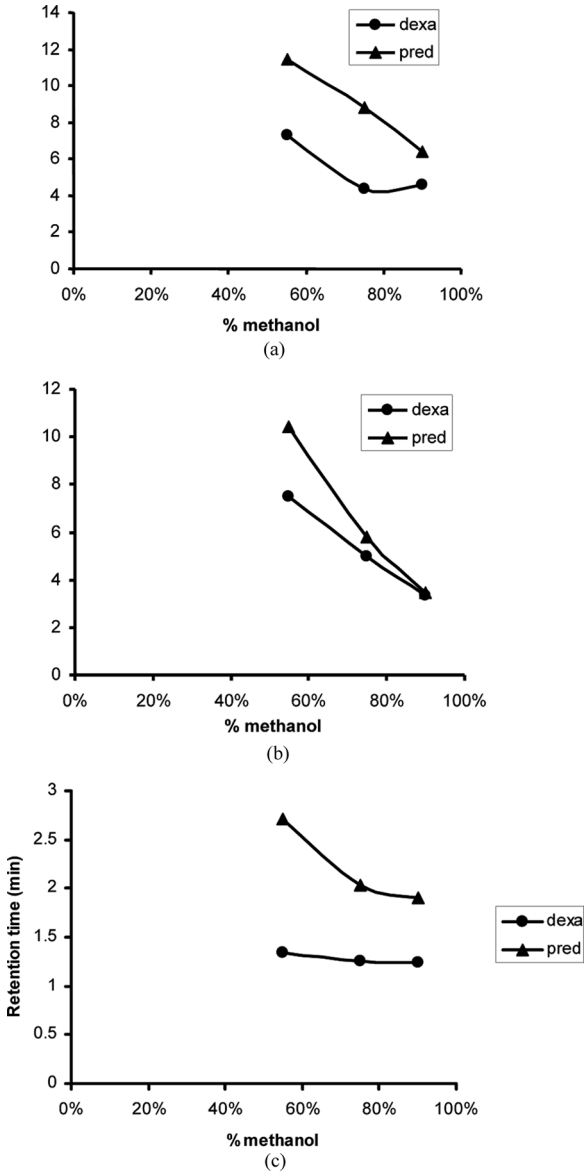

Figure 2. Plot of retention time of the cortisone compounds against the corresponding percentage of methanol using C_{18} (a), C8 (b), and CN (c) columns. Note that only the profile of DXSP (early eluting group) and PRD (late eluting group) are shown for clarity reasons.

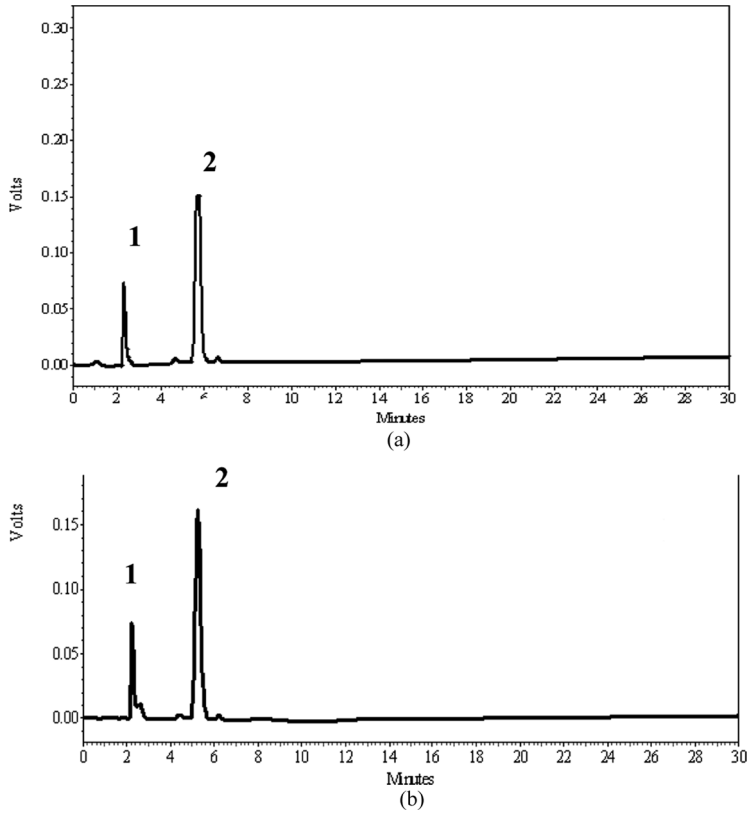

Figure 3. Representative chromatograms of the test cortisone compounds chromatographed on a C_{18} column using (a) the optimum mobile phase (75% methanol) and (b) the isoelutropic strength of acetonitrile (60%). Peak identification, 1 = BTSP and DXSP, 2 = PRD, HCA and FRM.

compounds; consequently, no improvement in the separation was noticed. In fact, the lack of selective effect of pH on the retention of the cortisone compounds was anticipated because the test compounds do not have ionizable groups that would be influenced by changing the value of pH in that range.

Chromatographic behaviors of the cortisone compounds were also characterized on a normal phase silica column. Methanol and dichloromethane were chosen as the strong and weak eluents, respectively. All the cortisone compounds appeared to have very little affinity towards the normal phase column using various combinations of methanol:dichloromethane as mobile phases. The longest retention time obtained was less than 3 min (DXSP and BTSP). It is noteworthy, however, that

the test compounds were clustered into two groups in a similar manner to what was observed using the reversed phase column, i.e., DXSP and BTSP eluted at the same retention time while HAC, PRD, and FRM eluted at another. Several previous studies have reported the successful separation of different cortisone compounds (albeit not the same combination used in this study) using normal phase chromatography.[8–11] In general, the reported methods relied on more lipohilic solvents such as hexane and heptane and/or the use of acetic acid. Therefore, the mobile phase combinations recommended in these reports have been tried to separate the five cortisone compounds in this study. None of the examined conditions provided a promising separation of the cortisone compounds. Consequently, and due to the aqueous nature of the mobile phases used with reversed phase mode, it was decided to continue method development using a reversed phase C_{18} column.

Using 75% methanol in water as the optimum mobile phase for the C_{18} column, efforts were made to improve the separation through using mobile phase additives. Mobile phase additives are known means by which the separation on reversed phase columns might be improved.[12–14] Additives such as urea (0.2 g/100 mL), ammonium acetate (0.2 g/100 mL), triethylamine (0.5 mL/100 mL), and hydroxy propyl B-cyclodextrin were examined. Overall, there were no significant changes in relative retention of the test compounds, i.e., the best result obtained was quite similar to the optimum obtained using the C_{18} column with 75% methanol without additives (see chromatogram in Figure 3).

To this end, it was necessary to investigate the effect of a solvent with a significantly different selectivity,[7] i.e., tetrahydrofuran (THF). A Previous study has reported successful separation of a group of cortisone compounds using a reversed phase column with 25% THF in water as a mobile phase.[15] Thus, the starting point was a mobile phase consisting of THF:H_2O (25%:75%). This mobile phase resulted in a reasonable separation of the late eluting cortisones (FRM, PRD, HCA). However the early eluting cortisones (DXSP, BTSP) remained unresolved (Figure 4). Because DXSP and BTSP are epimers, their separation on simple reversed phase packing was not anticipated. There were few reports describing separation of DXSP and BTSP on reversed phase HPLC. In most of these, no actual separation was accomplished, but the use of MS as a HPLC detector enabled differentiation between the two epimers as they exhibit subtle differences in their fragmentation patterns.[16–17] In one recent report the separation of the two compounds was achieved by incorporating organic modifiers (e.g., isopropanol) and surfactants in the mobile phase, which resulted in effectively long run times.[18] Nevertheless, the effects ofvarious mobile phases consisting of different percentages of methanol and THF were examined. In spite of some improvement in the resolution of the late eluting compounds, none

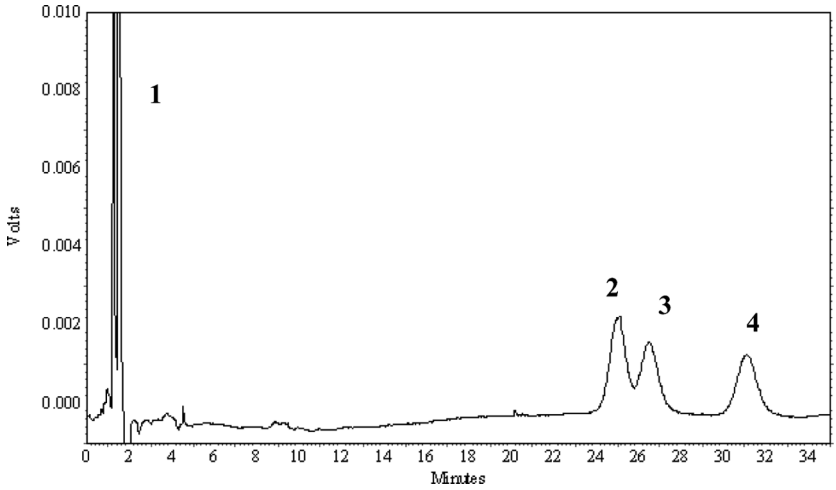

Figure 4. A representative chromatogram for the test cortisone compounds on a C_{18} column with a mobile phase consisted of 25% THF in water. Peak identification, 1 = BTSP and DXSP, 2 = PRD, 3 = HCA and 4 = FRM.

of the examined ratios resulted in any separation for DXSP and BTSP. The optimum of these conditions was concluded to be THF:methanol: water (15%:25%:60%). However, the achieved separation was less than desired because of very long retention for the late eluting (~30 min) compounds, and no separation was obtained for the early eluting ones (Figure 5).

At this point, the effects of sodium acetate and phosphoric acid on the chromatographic behavior of the cortisone compounds were examined. These were incorporated in the mobile phase at concentrations (0.025–0.1 M) while keeping the pH at 4.2. Addition of either sodium acetate or phospohoric acid, surprisingly, resulted in separation of DXSP from BTSP. Best resolution was obtained with phosphoric acid at concentration of 0.1 M (Figure 6). To the best of our knowledge, it was the first time where the separation of such epimeric compounds on reversed phase C_{18} columns was affected by the presence of simple buffer ions. Although a previous study reported the chromatographic separation of DXSP and BTSP, the reported separation was attributed to the use of surfactants together with organic modifiers such as propanol.[18] In this study, it appears that simple ions (mainly phosphate or acetate) bring about the otherwise unexpected separation.

In efforts to further cut the run time, additives like heptane sodium sulfate, and camphor sulfonic acid (CSA) were incorporated in the mobile phase hoping that they would selectively affect the retention of the different cortisone compounds. Only CSA (0.01 M) resulted in the

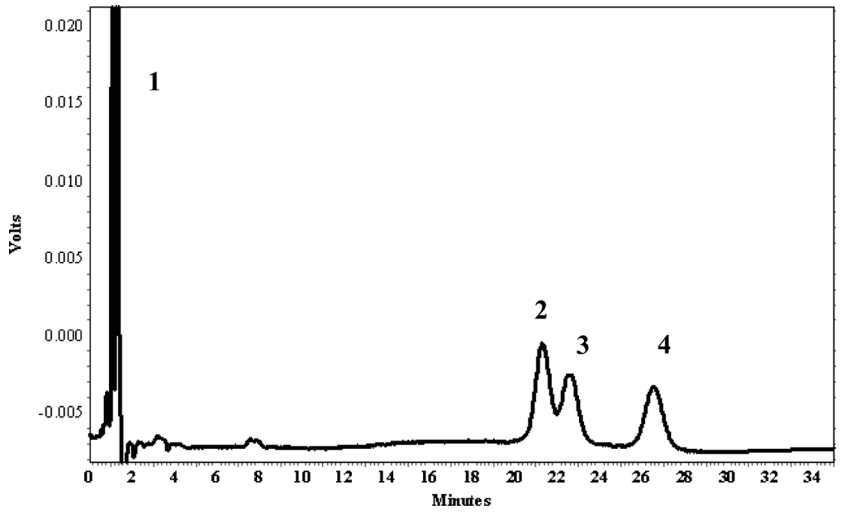

Figure 5. Representative chromatograms of the test cortisone compounds on a C_{18} column with a mobile phase consisting of THF:methanol:water (15%:25%:60%) at pH = 4.2.

desired result (Figure 7).It appeared that camphor sulfonic acid selectively occupied the more hydrophobic retention sites of the stationary phase leading to a lower chance of the late eluting peaks (more

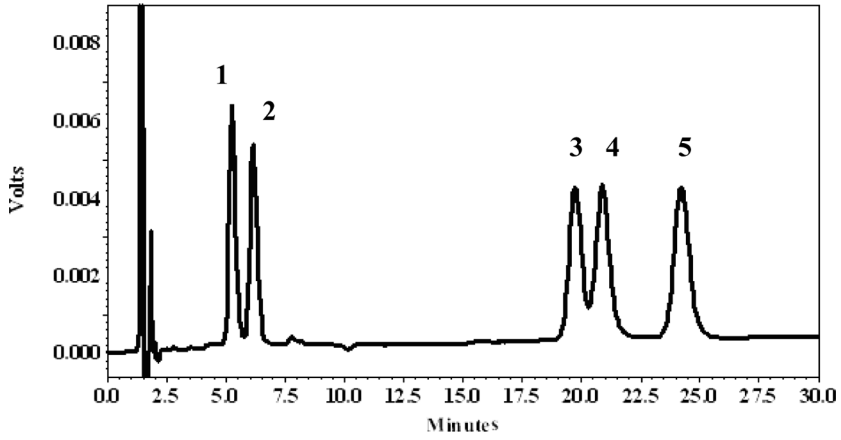

Figure 6. A representative chromatogram for the test cortisone compounds on a C_{18} column with a mobile phase consisted of THF:methanol:0.1 M phosphoric acid (15%:25%:60%) at pH = 4.2. Peak identification, 1 = BTSP, 2 = DXSP, 3 = PRD, 4 = HCA and 5 = FRM.

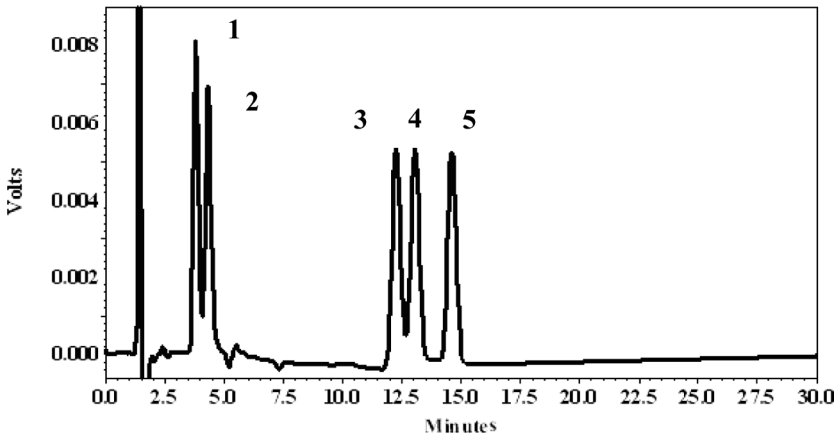

Figure 7. A representative chromatogram for the test cortisone compounds on a C_{18} column with a mobile phase consisted of THF:methanol:0.1 M phosphoric acid (15%:25%:60%) at pH = 4.2 containing 0.01 M CSA. Peak identification, 1 = BTSP, 2 = DXSP, 3 = PRD, 4 = HCA and 5 = FRM.

hydrophobic) to interact with the stationary phase. Consequently, the late eluting compounds would be more seriously affected, i.e., their retention times would be dramatically decreased while the retention times of the less hydrophobic compounds would be only slightly changed (compare chromatograms in Figures 7 and 6). Overall the total run time was shortened from about 30 min to 15 min while the resolution maintained at almost the same level.

Effect of pH

While keeping the mobile phase composition constant (the concluded optimum in the previous section, i.e., 15% of THF:25% of methanol:60% of 0.01 M phosphoric acid containing 0.01 M CSA), the pH was varied in the range (3.0–5.5). In general, all cortisone compounds exhibited broader peaks and longer retention times at lower values of pH with FRM being the most seriously affected. However, there were only subtle changes in resolution, and pH value of 4.2 was decided as the optimum.

Effect of Temperature

The temperature of the column was changed in the range (25–40°C) and the cortisone mixture was chromatographed using the optimum mobile

phase concluded in the previous section, i.e., THF 15%: methanol 25%: 0.01 M phosphoric acid 60% containing 0.01M CSA, pH = 4.2. The plot of the retention time for each compound against the corresponding temperature (Figure 8) showed that retention decreased with increasing temperature. However, temperature did not appear to have a selective effect on the various cortisones so that it did not seriously manipulate the resolution of the test mixture. It was also noteworthy to observe that the peaks of the various cortisone compounds became broader with increasing temperature, which was rather dissimilar to many analytes. Therefore, 25°C was taken as the optimum temperature for separation. Thus, the overall recommended conditions for the proposed method were a mobile phase that consisted of THF:methanol:0.01 M phosphoric acid at a percentage of 15:25:60 containing 0.01 M camphor sulfonic acid with pH adjusted to 4.2. At this condition, all cortisone compounds were sufficiently separated to be detected and identified in a cross contaminated TM eye drop preparation. In practice, if a TM eye drop preparation was contaminated then most likely the contaminant would be only one of the potential cortisone compounds. Thus, accurate quantification would be enabled.

To this end, the proposed method was subjected to an extensive validation procedure according to internationally accepted guidelines.[19,20]

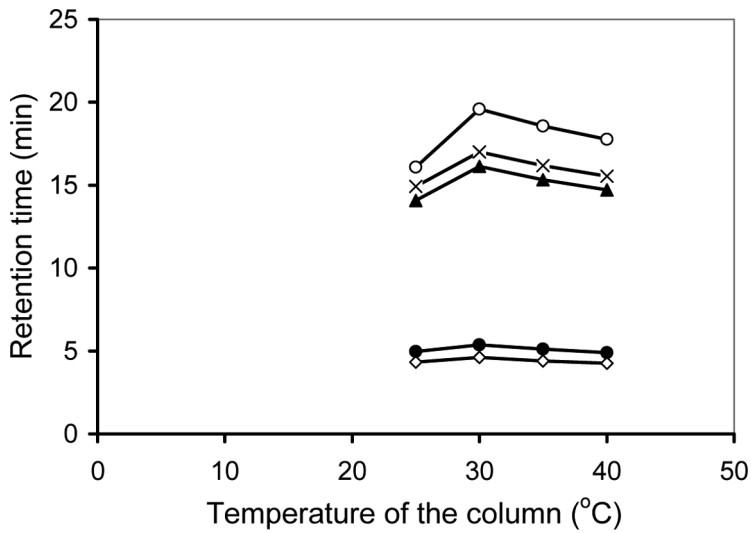

Figure 8. A plot of retention times for the different cortisone compounds against temperature of the column, other chromatographic conditions as in Figure 7. BTSP = ◇, DXSP = ●, PRD = ▲, HCA = ×, and FRM = o.

Validation and Application of the Proposed Method

Linearity

Nine standard solutions having increasing concentration of each compound were prepared as described in experimental section, calibration curves were constructed for each compound in the specified concentration range (0.04–100 μg/mL). Five injections were made at each concentration level and the average value was reported. The linearity was assessed using the least square method. The data presented in Table 1 indicate good linearity for all of the examined cortisone compounds over the studied concentration range.

Precision

Five determinations of each compound at the lowest, middle, and highest concentration levels were performed. The obtained data were presented in Table 2. According to the shown RSD values, the method was concluded to be precise.

Sensitivity

The limit of quantification (LOQ) was accepted as 40 ng/mL since it is the lowest concentration examined in this study and provided an acceptable RSD valueless than 1 (ICH Q2A, 1994). More over, the signal to noise ratio was higher than 15. The limit of detection (LOD) for the various cortisone compounds was taken as 10 ng/mL because it provided a signal to noise ratio of 4. Overall, the demonstrated sensitivity limits were quite sufficient to detect and quantify potential contamination with cortisone compounds. Although there was no predetermined limit for the concentration of the potential cortisone contaminants in an eyedrop, a minimum of 40 ng/mL was considered satisfactory because it represents 100–1000 times dilution of the pharmacologically effective cortisone preparation.

Table 1. Calibration equations for the examined cortisone compounds. Reported values are the average of five determinations.

Cortisone compound	Average equation	R^2
DXSP	Y = 0.033X+252.42	0.9994
BTSP	Y = 0.0343X+228.01	0.9998
HCA	Y = 0.027X–32.35	0.9999
PRD	Y = 0.0257X+117.44	0.9998
FRM	Y = 0.022X+6.3576	0.9999

Table 2. Precision data for the various cortisone compounds at three concentration levels (n = 5)

Cortisone compound	RSD lowest	RSD middle	RSD highest
DXSP	0.197261	0.700866	0.80308
BTSP	0.70804	0.724824	0.84756
HCA	0.386143	0.5578	0.9856
PRD	0.614269	0.88603	0.95214
FRM	0.463346	0.6360063	0.816962

Selectivity

The selectivity of the method was ensured by subjecting all potential excipients that might be present in the eye drop preparation (see experimental) to the same chromatographic procedure of the proposed method. Resulting chromatograms demonstrated lack of any interference from the studied cortisone compounds. Timolol maleate was shown to elute very early in the chromatogram (away from cortisone compounds) because it is completely ionized in the employed acidic mobile phase. Moreover, the potential interference from the likely degradation products of timolol was also assessed. Timolol solutions that were exposed to degradation using HCl, NaOH, and H_2O showed newly emerging peaks when chromatographed using the proposed condition. No serious overlapping with the peaks of the analytes was observed (Figure 9). Therefore, it was concluded that the proposed method was selective enough for the purpose of detection and quantification of potential cortisone compounds in eye drop preparations.

Ruggedness

Table 3 shows that varying the temperature within two degrees above and below the recommended value has almost insignificant effect on the retention time (RSD for obtained retention times were less than 2.1). Changing the percentage of methanol within the range 23–27% also appears not to significantly influence the retention time of the test compounds (RSD less than 1.5). The effect of changes in pH on the retention times of the test compounds was modest with DXSP and BTSP being the most seriously affected. However, changing the percentage of THF within the range (13–17%) appeared to have the most serious effect on the retention time of the five analytes (RSD $\sim$ 13–16). In practice, it is unlikely to have such a serious change in percentage of THF (+/−13% of the optimum) if reasonable care was taken during preparation of the

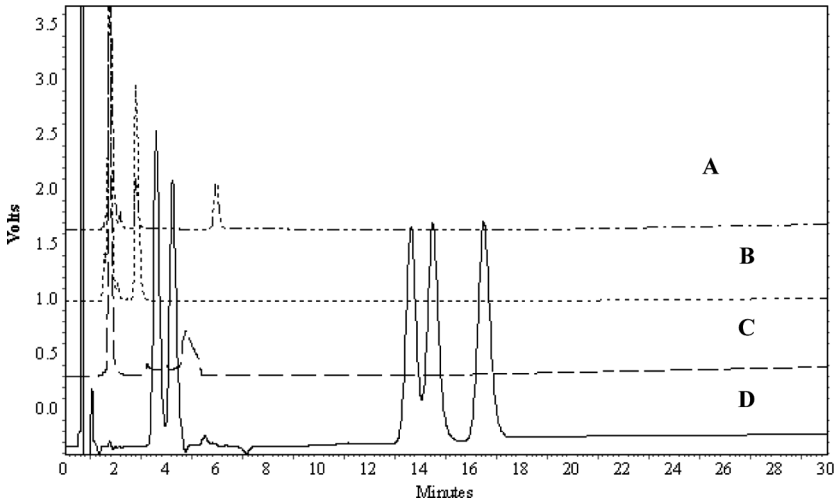

Figure 9. Overlaid chromatograms for the degradation solution of timolol maleate in NaOH (A), H_2O_2 (B), HCl (C) and standard mixture of cortisone compounds (D). The first eluting peak in chromatograms A, B and C corresponds to TM. Peaks of cortisone are identified as in Figure 7.

mobile phase. Nevertheless, this was not found to affect the performance of the method in detecting presence of potential cortisone compounds in eye drops, because a standard solution of the five cortisone compounds should be run along with the test sample using the same mobile phase.

Application to Commercial Timolol Eye Drops

The method was applied for detection of potential cortisone compounds in the timolol maleate eye drop preparation. Five samples that were collected from the local market were injected directly onto the HPLC

Table 3. RSD values for the retention times of the cortisone compounds when obtained at conditions slightly different from the recommended optimum

RSD values Parameter	BTSP	DXSP	PRD	HCA	FRM
Temperature (23–27°C)	0.74	2.1	0.25	0.74	0.18
pH (4.0–4.5)	4.46	4.62	1.76	1.66	2.81
% methanol (23–27%)	1.2	0.86	0.31	1.24	1.18
% THF (13–17%)	16	14.46	13.91	14.36	16.14

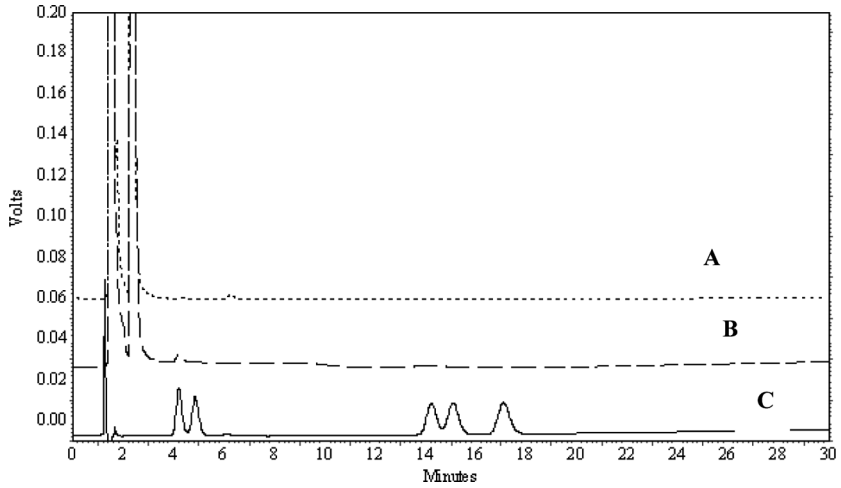

Figure 10. Overlaid chromatograms for the assayed commercial samples (A + B) along with that of standard cortisone mixture (C).

system and assayed using the proposed method. The results showed that only one eye drop preparation (Timolol 0.5%) from the five assayed samples suggested the presence of BTSP in the commercial sample. However the estimated level of BTSP = 1.78 μg/mL. Knowing that the lowest pharmaceutical dose of BTSP eye drops is 1 mg/mL then the found level (1.78) is almost 560 times less than the pharmacologically effective dose.

In conclusion, the chromatographic behavior of five cortisone compounds have been characterized and optimum condition for their separation was achieved. To the best of our knowledge it was the first time where the separation of DXSP and BTSP was shown to be influenced and achieved by simple ions. The developed method was shown to be valid for detection and quantification of cortisone residues in timolol maleate eye drops. Application of the method to commercially available samples revealed a detectable level of BTSP in one out of five preparations tested.

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