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Analysis of an anti-inflammatory steroidal drug, difluprednate, in aqueous humor by combination of semi-micro HPLC and column switching method

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

A specific and sensitive method for the determination for difluprednate (DFBA) and its metabolite (deacetylated DFBA, DFB) in aqueous humor was developed. DFBA and DFB were initially absorbed on a Pinkerton-type column, then analyzed by high-performance liquid chromatography using a semi-micro column after column switching. Under the optimized conditions, calibration curves for DFBA and DFB showed good linearity over the range of 1.0–50 and 0.5–50 ng/ml, respectively. We applied the method to the analysis of DFBA and DFB in rabbit aqueous humor, and found that DFBA in rabbit aqueous humor 1 h after instillation of 0.002% DFBA ophthalmic emulsion was not detected, but DFB was present at the concentration of 4.3 ± 3.1 ng/ml.

Keywords: Semi-micro HPLC; Column switching method; On-line concentration; Difluprednate; Aqueous humor

1. Introduction

Analysis of pharmaceuticals and their metabolites in biological fluids by high-performance liquid chromatography (HPLC) includes various difficulties due to many concomitants such as proteins, lipids and salts in the sample solution. Prior to the analysis by HPLC, we often carry out pretreatment procedures such as deproteinization, extraction and pre-concentration in order to improve separation efficiency and reproducibility. However, these procedures are usually tedious and time-consuming, and often cause low recovery.

The "in-line" deproteinization method by HPLC has been developed to allow direct injection of plasma samples [1–6], and the use of a Pinkerton-type stationary phase has become one of the good strategies for the analysis of pharma-

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ceuticals in biological fluids. Pharmaceuticals in the biological samples are trapped in the pretreatment column such as a Pinkerton-type column and contaminant components such as proteins are excluded. The trapped pharmaceuticals are then introduced to the concentration column, and then to the analytical column by column switching method [7–12].

The ophthalmic product including a steroidal anti-inflammatory component such as betamethasone sodium phosphate is often used for treatment of inflammation, but its instillation may cause the ocular hypertension. A synthetic glucocorticoid medicine, difluprednate (DFBA, 6α,9-difluoro-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione 17-butyrate 21-acetate), is also used for the treatment of inflammation (see Fig. 1) [13]. DFB, a deacetylated metabolite of DFBA, also shows similar activity to that of DFBA [14]. DFB is further metabolized to the compound which is a hydroxylated form of DFB at C-6, 9-fluoro-6β,11β,17,21-trihydroxypregna-1,4-diene-3,20-dione 17-butyrate (HFB). HFB does not show the activity.

It is important to investigate the ocular absorption of drugs after instillation of ophthalmic product in order to understand the effect as well as the side effect. This study is quite important for the appropriate use of DFBA as ophthalmic emulsion. Unfortunately, direct analysis of the aqueous humor often encounters difficulty in determination of pharmaceuticals due to the extremely low concentrations and contaminant protein components. Therefore, we have to carry out the procedures for pre-concentration of pharmaceuticals and removal of protein contaminants. For these reasons, this report describes a method for simultaneous determination of DFBA and DFB in aqueous humor using a combination of semi-micro HPLC and column switching method.

2. Experimental section

2.1. Materials

Samples of DFBA and DFB were supplied by Mitsubishi Pharma Corporation (Chuo-ku, Osaka, Japan). Acetonitrile (HPLC grade) was purchased from Nacalai Tesque, Inc. (Nakagyo-ku, Kyoto, Japan). The ophthalmic emulsion of DFBA was prepared by the manufacturer, and contained isotonic agent, buffering agent and preservatives. Water was purified with a Milli-Q purification system (Millipore, Shinagawa-ku, Tokyo, Japan) after deionization of water. Other reagents were HPLC grade or the highest grade commercially available. Male Nippon albino rabbits (about 2 kg) were used in the present study, and from Japan Laboratory Animals Inc. (Nerima-ku, Tokyo, Japan). Standard solutions of DFBA and DFB were prepared by dissolving each of them (2.0 mg) in acetonitrile (1.0 ml). A portion of each solution was mixed and diluted with water.

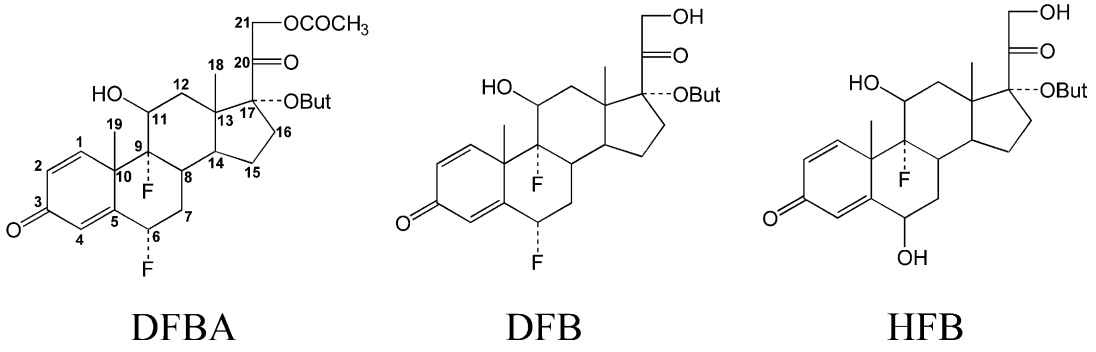

Fig. 1. DFBA and its metabolites. Difluprednate (DFBA): 6α, 9-difluoro-11β, 17, 21-trihydroxpregna-1, 4-diene-3, 20-dione 17-butyrate 21-acetate, DFB: 6α, 9-difluoro-11β, 17, 21-trihydroxpregna-1, 4-diene-3, 20-dione 17-butyrate and HFB: 9-fluoro-6β, 11β, 17, 21-trihydroxypregna-1, 4-dine-3, 20-dione 17-butyrate.

2.2. High-performance liquid chromatography (HPLC)

2.2.1. Conventional method

An HPLC system (TOSOH 8020, Tosoh, Minato-Ku, Tokyo, Japan) was composed of an autosampler (AS-8020), a pump (DP-8020), a column oven (CO-8020), a UV detector (UV-8020) and a data processing software (LC-8020) with an octadecylsilica column (TSK-gel ODS-80Ts, 150-mm, 4.6-mm i.d., Tosoh) was used. Analysis of DFBA and DFB was carried out using a mixture of 10 mM phosphate buffer (pH 3.0) and acetonitrile (60:40 v/v%) as mobile phase at a flow-rate of 0.8 ml/min at 40 °C. Detection was performed at 240 nm. Solutions of DFB at various concentrations were prepared by diluting the standard solution (see above) with acetonitrile–water (1:1 by volume). The injection volume was 50 μl.

2.2.2. Semi-micro method

A semi-micro HPLC system (Nanospace SI-1, Shiseido, Chuo-ku, Tokyo, Japan) was composed of an autosampler (Type 2023), a pump (Type 2001), a column oven (Type 2014), a UV detector (Type 2002) and a data processing system (S-Micro Chrom Ver4.1). The system used in the present study is illustrated in Fig. 2.

A Capcell Pak MF cartridge pH-1 column (**4**, Shiseido, 10-mm, 4.0-mm i.d.) was used for selective adsorption of DFBA and DFB in aqueous humor. This column was packed with polymer-coated silica having hydrophilic polyoxyethylene groups on the surface and hydrophobic phenyl groups in the pore. A Capcell Pak C18 UG80 S5 column (**6**, Shiseido, 35-mm, 1.5-mm i.d.) was used for concentration of the analytes. A Capcell Pak C18 UG120 S5 column (**7**, Shiseido, 250-mm, 1.5-mm i.d.) was used as the separation column. The operation temperature was kept at 40 °C during analysis. However, pretreatment and concentration columns were operated at room temperature.

Pumps **1** and **2** were used to deliver eluent 1 [10 mM phosphate buffer (pH3.0) containing 40 v/v% of acetonitrile] at a flow rate of 0.1 ml/min and eluent 2 [10 mM phosphate buffer (pH3.0) containing 15 v/v% of acetonitrile] at a flow rate of 0.2 ml/min, respectively. Detection was performed at 240 nm.

Time-schedule and other conditions for the analysis of DFBA and DFB in aqueous humor are summarized in Fig. 3. In the initial step, a sample solution (150 μl) was introduced to the pretreatment column (**4**) via the autosampler (**3**) (Fig. 2A) using eluent 2. The pretreatment column keeps hydrophobic DFBA and DFB. Proteins in aqueous humor are eluted in void volume, and wasted without passing through the analytical column (**7**) and the detector (**9**). DFB is eluted from the pretreatment column between 6.0 and 9.3 min, and introduced to the concentration column (**6**) by switching the valve as shown in Fig. 2B (Step 2). The eluate between 9.3 and 15.0 min does not include any metabolites, and is wasted by

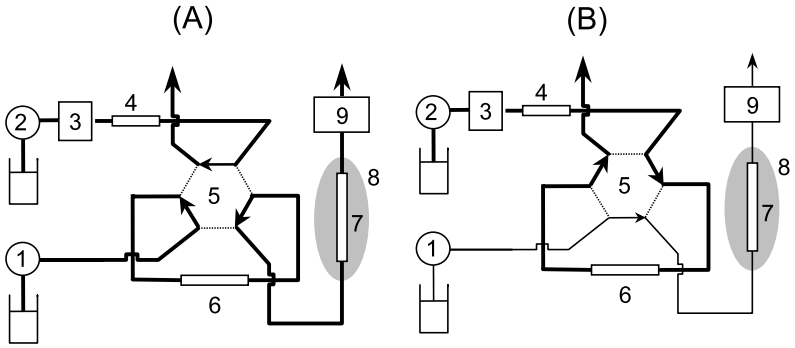

Fig. 2. Schematic diagram of HPLC: (A) Removal position of proteins and separation position. (B) Concentration position. 1, pump (for separation); 2, pump (for pretreatment); 3, autosampler; 4, pretreatment column; 5, switching valve; 6, concentration column; 7, analytical column; 8, column oven; 9, UV detector.

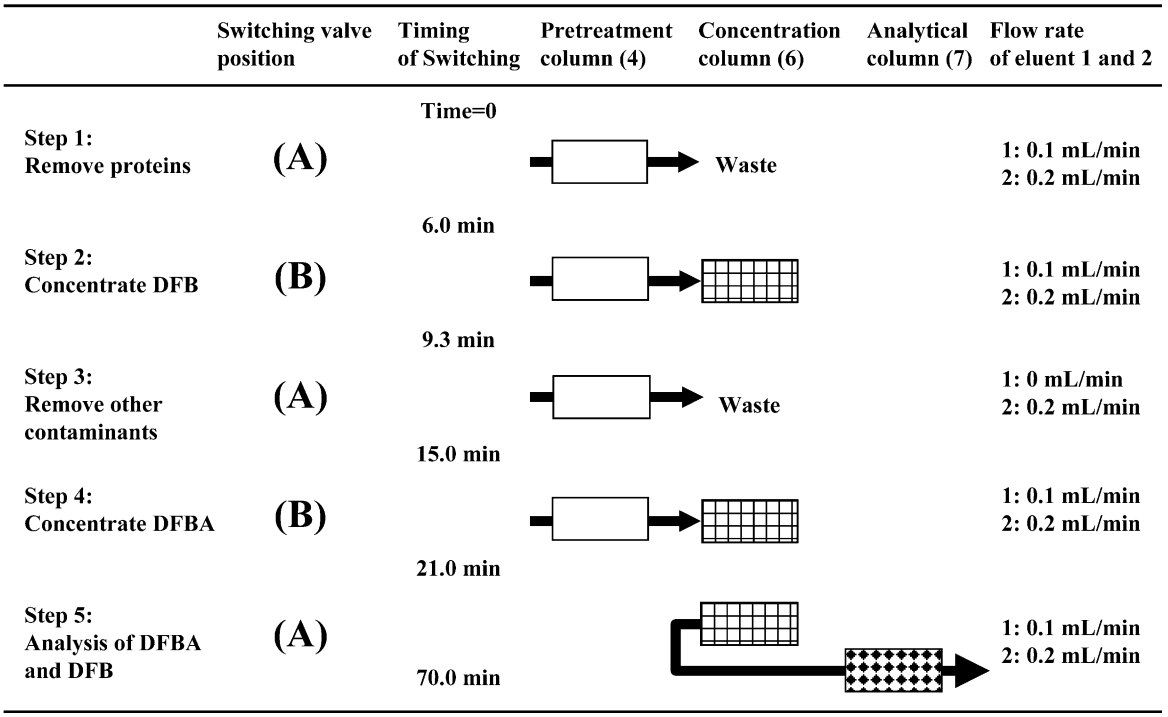

Fig. 3. Time-schedule of column switching for the analysis of DFBA and DFB in aqueous humor.

switching the valve as shown in Fig. 2A. To inhibit the movement of DFB in the concentration column during this period, pump 1 is stopped (Step 3). The other analyte, DFBA, is then eluted from the pretreatment column between 15.0 and 21.0 min, and introduced to the concentration column (**6**) by switching the valve as shown in Fig. 2B. Finally DFBA and DFB adsorbed in the concentration column are introduced to the separation column (**7**) by switching the valve as shown in Fig. 2A with back-flush technique [9] using eluent 1. During the analysis, the pretreatment column is washed for the next analysis (step 5).

2.3. Hydrolysis of DFBA in aqueous humor

The samples of the aqueous humor, to which previously were added DFBA at 100 ng/ml, were incubated at room temperature. The concentration of DBFA and DFB in the samples after 0, 10, 20, 30 and 60 min were assayed by the conventional HPLC. The experiments were performed in triplicate using three sample solutions.

2.4. In vivo ocular absorption

Unanesthetized rabbits were kept in a prone position on a stainless plate. An ophthalmic emulsion of DFBA (50 μl) was instilled directly onto the corneas of rabbits. A rabbit was killed by intravenous overdose administration of sodium pentobarbital solution 1 h after instillation, and the aqueous humor (about 200 μl) was collected with a syringe after the eye was washed with saline. A portion (150 μl) of the sample was analyzed by HPLC as described above. All experimental procedures were approved by the institutional Committee for the Care and Use of Laboratory Animals.

3. Results and discussion

3.1. Analysis of DFB in standard solutions by conventional HPLC

The results for the analysis of DFB in standard solutions at various concentrations are shown in Fig. 4. DFB was observed at 13 min. Calibration curves showed good linearity above the concentration of 10 ng/ml. The equation for the regression analysis was $y = 0.074x - 0.396$ ($R = 1.00$), where y is the response (mV/s) and x is the concentration of DFB (ng/ml). Lower limit of detection of DFB was approximately 5 ng/ml at the level of S/N = 2. Because we have to determine at least a few ng/ml of DFB in aqueous humor, optimization studies on the semi-micro HPLC method were performed.

3.2. Elution profile of DFBA and DFB in rabbit aqueous humor on the pretreatment column

As the preliminary studies on the analytical conditions for clean-up procedures, a sample (150 μl) of the aqueous humor was analyzed using the pretreatment column without installing the analytical column. A sample (150 μl) of the aqueous humor containing DFBA and DFB at concentration of 100 ng/ml was injected to the pretreatment column. The separation was shown in Fig. 5.

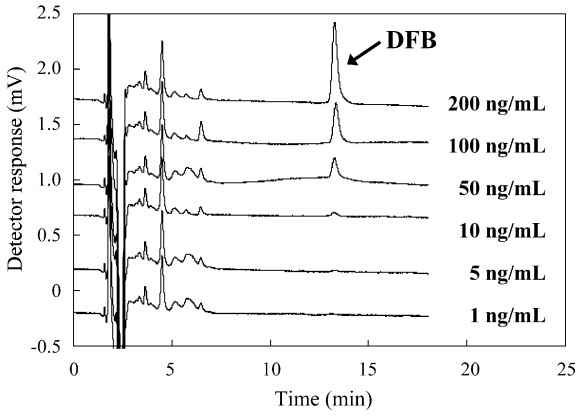

Fig. 4. Analysis of the standard solutions of DFB at various concentrations using conventional HPLC.

Huge peaks due to proteins were observed at earlier elution time within 6 min. DFB (the deacetylated product of DFBA) was observed around 7.5 min. DFBA, on the contrary, was retarded and observed around 17 min. From these results, we devised the program for the analysis of these compounds as shown in Fig. 3. In step 1, the eluate containing proteins was wasted, then the eluate including DFB was introduced to the concentration column from 6.0 to 9.3 min in step 2. The eluate from 9.3 to 15.0 min was wasted in step 3. It should be noticed that the flow in the concentration column was stopped during this period in order to avoid leakage of DFB from the concentration column. Finally, DFBA and DFB retained on the concentration column were eluted and introduced to the analysis column. We changed the flow direction of the concentration column as shown in step 5. Back-flush technique (changing the flow) made it possible to shorten the analysis time. Sharpening the peaks and increasing the recovery were also performed by this technique [9].

3.3. Column-switching system

We analyzed standard of the DFBA and DFB using the column switching method. In the present mode of separation, the calibration curves showed linear relationships between 1.0–50 ng/ml for DFBA and 0.5–50 ng/ml for DFB. The results are shown in Fig. 6. The equations for the regression analysis were $y = 1.28x - 0.41$ ($R = 1.00$) for DFBA and $y = 1.21x + 0.51$ ($R = 1.00$) for DFB, respectively, where y is the response (mV/s) and x is the concentration of DFBA and DFB (ng/ml). The relative standard deviations in determination of DFBA and DFB were 2.9, 2.7, 1.7 and 1.3% at 1.0, 5.0, 10 and 50 ng/ml for DFBA, and 5.2, 10.4, 0.9, 0.8 and 1.7% at 0.5, 1.0, 5.0, 10 and 50 ng/ml for DFB, respectively. A peak was observed around 45 min. At present, we could not confirm it, but the peak was due to a contaminant of the standard sample of DFB. Although we did not estimate the leaking of DFBA and DFB from the concentration column during the operation of concentration, the results on the overall recovery showed almost quantita-

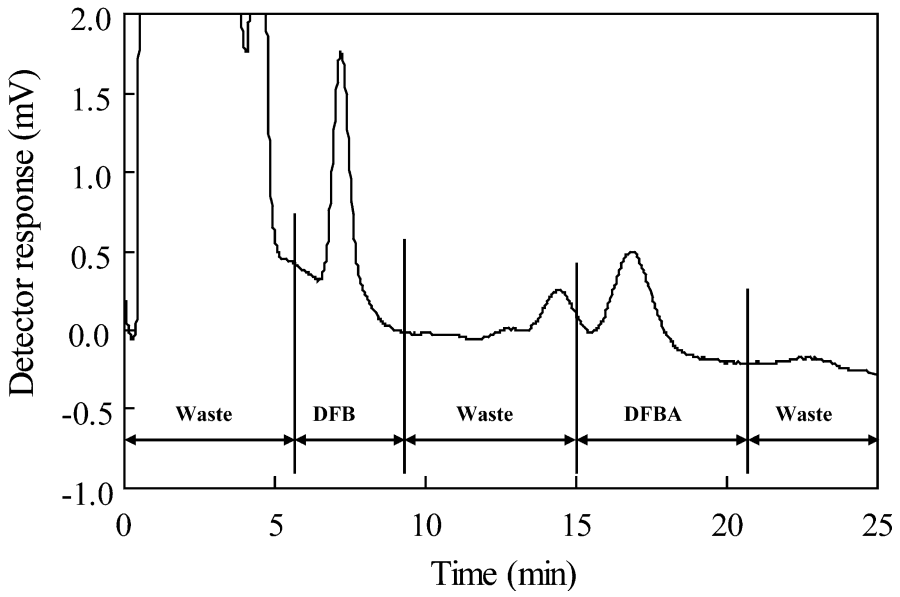

Fig. 5. Separation of DFBA and DFB spiked in the aqueous humor at the concentrations of 100 ng/ml for both compounds using the pretreatment column.

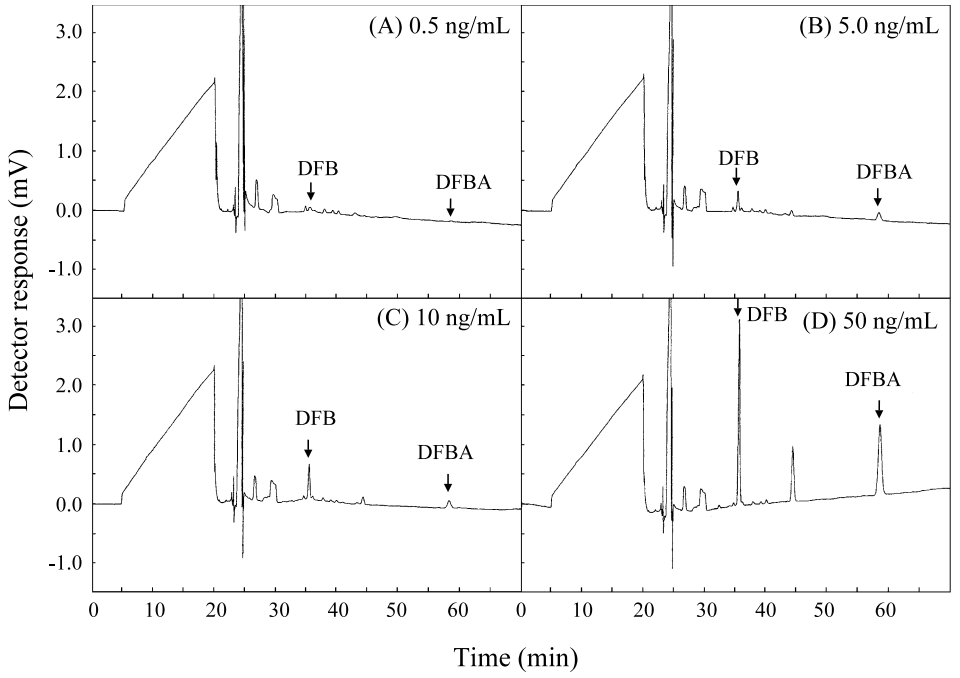

Fig. 6. Analysis of standard solutions of DFBA and DFB at different concentrations using column switching technique.

tive recovery. Also, there were no carry over between injections.

To determine the effect of partial hydrolysis of DFBA on the analysis of DFBA and DFB, we added DFBA to the aqueous humor at 100 ng/ml, and incubated at room temperature. A portion of the mixture was analyzed at specified intervals. The results are shown in Fig. 7. Concentration of DFBA gradually decreased with time, and about a third of DFBA (28 ng/ml) was determined 1 h after addition. On the contrary, concentrations of DFB gradually increased. Total concentrations of DFBA and DFB showed almost constant values during incubation. These data indicated that hydrolysis of DFB to other metabolites such as HFB (see Fig. 1) was negligible. Furthermore, we examined the recovery of DFB in the aqueous humor at 1 and 10 ng/ml, and found that the recoveries were 107±3 and 103±3% for 1 and 10 ng/ml, respectively. These data also support that further hydrolysis of DFB did not occur in the aqueous humor.

3.4. Analysis of DFBA and DFB after in vivo ocular absorption

DFBA and DFB concentrations in rabbit aqueous humor were determined 1 h after instillation of 0.002, 0.01 and 0.05% DFBA ophthalmic emulsion. The results are summarized in Table 1. DFBA after instillation of DFBA ophthalmic emulsion even at 0.05% concentration could not

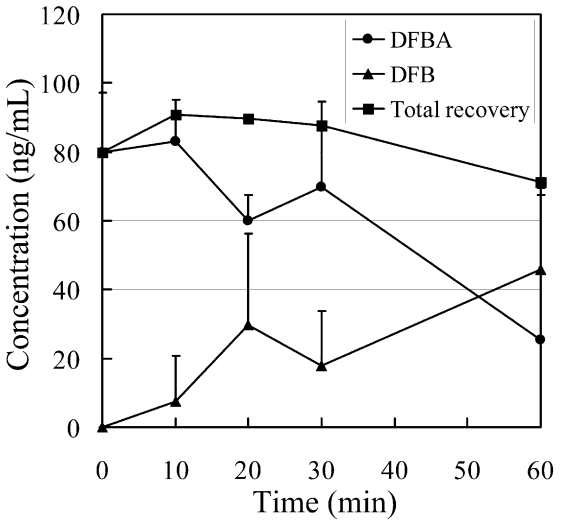

Fig. 7. Hydrolysis of DFBA to DFB during incubation in the aqueous humor.

Table 1
In vivo analysis of DFBA and DFB in the aqueous humor 1 h after instillation

Ophthalmic emulsion	Concentration (ng/ml)	
	DFBA	DFB
0.002% DFBA	N.D.	4.3±3.1
0.01% DFBA	N.D.	16.6±11.6
0.05% DFBA	N.D.	90.9±51.0

Each value represents mean±S.D. ($n=9$–10). N.D.: not detected.

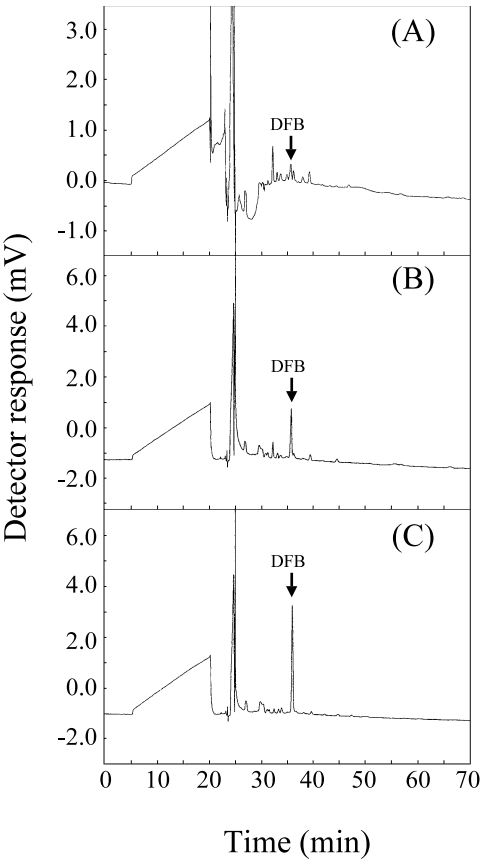

Fig. 8. Analysis of DFBA and DFB in the aqueous humor after in vivo addition of DFBA ophthalmic emulsion of (A) 0.002, (B) 0.01 and (C) 0.05%.

be detected (Fig. 8). On the contrary, DFB concentrations after instillation of 0.002, 0.01 and 0.05% DFBA ophthalmic emulsion were 4.3 ± 3.1, 16.6 ± 11.6 and 90.9 ± 51.0 ng/ml, respectively. These data mean that hydrolysis of the acetyl group of DFBA proceeds quite quickly.

In the present study, we reported the transfer of medicine to the aqueous humor in instillation of ophthalmic product using DFBA preparation. The sample volume of the aqueous humor was extremely small (about 200 μl) in rabbit. Although DFBA used in the present study is easily hydrolyzed to DFB and HFB in blood [14], we could not examine the presence of HFB in the aqueous humor of rabbit due to the extremely small volume (200 μl) of the sample. It seems important to analyze the metabolites of DFBA in the aqueous humor when it is used for ophthalmic product. We found that DFBA is easily changed to its deacetylated product and further hydrolysis does not occur within 1 h in aqueous humor. A combination of semi-micro HPLC and column switching technique is a good strategy for the analysis of pharmaceuticals in aqueous humor.

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