INTRAOCULAR FATE OF DEXAMETHASONE DISODIUM PHOSPHATE TOPICALLY APPLIED TO THE EYES OF RABBITS

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ABSTRACT

After instillation of ^{3}H-dexamethasone into the eyes of a rabbit, ^{3}H-9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione was found in the aqueous humor. The same metabolite was also formed by incubating ^{3}H-dexamethasone with the anterior ocular tissues of rabbit. Identification of ^{3}H-9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione was performed by its mobility on a thin layer plate and by proving its radiochemical homogeneity after recrystallization with the unlabeled sample which had been synthesized from dexamethasone by oxidation with sodium bismuthate.

When dexamethasone disodium phosphate was instilled into rabbit's eyes, it was hydrolyzed to free dexamethasone and then metabolized to 9α-fluro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione.

INTRODUCTION

In ophthalmology, synthetic corticosteroids (for example, dexamethasone, betamethasone, and fluorometholone) are used for local administration such as topical application to the eyes, subconjunctival injection and retrobulbar injection as well as systemic administration, and their excellent effect is well recognized clinically. However, it should be pointed out that unfavorable side effects often arise by the use of these agents. Among these side effects, steroid glaucoma is especially noteworthy.

The clinical symptoms of steroid glaucoma are very similar to those of primary open-angle glaucoma, and studies of steroid glaucoma might give information toward the elucidation of the pathogenesis of primary open-angle glaucoma.

Reports on the fate of synthetic corticosteroids topically applied to eyes are scanty. Krupin et al (1) found that dexamethasone disodium phosphate instilled into the eyes of rabbits appeared in the aqueous humor as such, or as free dexamethasone. Yamauchi et al.(2) observed an unidentified metabolite as well as ^{3}H-dexamethasone in the aqueous humor and the cornea of rabbits which had had ^{3}H-dexamethasone topically administered to the eyes.

The purpose of this experiment was to clarify the intraocular fate of dexamethasone disodium phosphate which is used frequently in collyria.

EXPERIMENTAL MATERIALS AND METHODS

[6,7-^{3}H] Dexamethasone(specific activity 33.0 Ci/m mol.) was purchased from the New England Nuclear Corp., and its radio-chemical purity was confirmed by thin layer chromatography. Non-radioactive dexamethasone and dexamethasone disodium phosphate were acquired from the Nippon Merck Banyu Co., Ltd.

Reagents and organic solvents used in the present study were of analytical grade and purified by distillation before use.

Synthesis of 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione

Dexamethasone was oxidized in acetic acid-H_2O with sodium bismuthate according to the method of Herzog et al. (3). Reaction products were extracted with ethyl acetate and after removal of the solvent the residue was dissolved in benzene/ethyl acetate 9:1(V/V) and applied on a column of silica gel prepared in benzene. The column was eluted with benzene-ethyl acetate 9:1, 8:2, and 7:3(V/V), succes-

sively. The material eluted with benzene-ethyl acetate 7:3 (V/V) was recrystallized with acetone-n-hexane to give crystals of mp 214 °C(uncorr.); max 238 mμ.

The substance showed only one spot by thin layer chgomatography and the mass spectrum (Fig. 1), as well as the Fourier-transfer ^{13}C-NMR spectrum (Fig. 2), confirming that it was 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione (4).

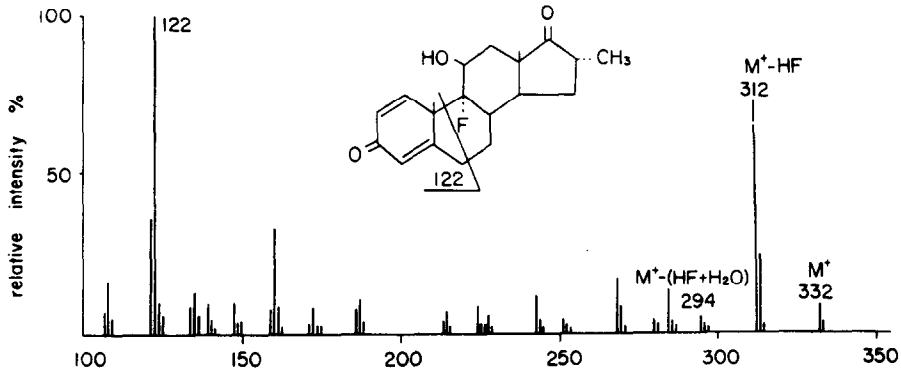

Fig. 1 Mass spectrum (above m/e 100) of 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione.

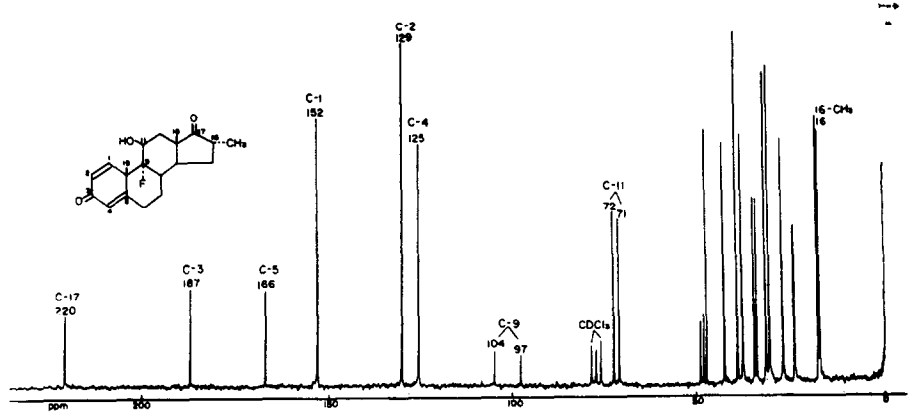

Fig. 2 Proton noise decoupled Fourier-transform ^{13}C-NMR-spectrum of 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione.

Ultraviolet absorption spectrum was determined by the Hitachi Perkin Elmer UV-VIS spectrometer. Mass spectrum was taken with JMS-OISG mass spectrometer. Proton noise decoupled ^{13}C-FT NMR and off-resonance decoupled ^{13}C-FT NMR were taken by Dr. E. Mizuta of the Research Laboratories, Takeda Chemical Industries Ltd.

Preparation of ^{3}H-dexamethasone solution used for collyria

[6,7-^{3}H] Dexamethasone was dissolved in 2 ml of acetone containing 20 mg of propylene glycol. After the acetone was evaporated under N_2 gas, the residue was dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.4) containing 100 µg of benzalkonium chloride.

The ^{3}H-dexamethasone solution contained 0.5 µCi of ^{3}H-dexamethasone per 1 µl.

The non-radioactive dexamethasone disodium phosphate solution (5 mg/ml) was prepared by increasing the content of dexamethasone disodium phosphate by addition of dexamethasone disodium phosphate to a commercial dexamethasone disodium phosphate solution (Decadron).

Topical administration and collection of samples

Eighty µl of ^{3}H-dexamethasone solution, in total, was instilled into the eyes of mature white rabbits (weighing 2.5-3 kg) anesthetized with 20% of urethane.

There were made four applications of 20 µl, each topically applied at three min. intervals. Fifteen min. after the last administration, a total of 0.6 ml of the aqueous humor was collected from the eyes by puncture of the anterior chamber via the sclera. After collection of the aqueous humor, both eyeballs were immediately excised, washed with physiological saline solution and analyzed. Forty ml of blood was collected by cardiac puncture, and these sera were also analyzed.

Incubation

^{3}H-dexamethasone (5 µCi) was incubated at 37°C for 1 hr. in 6 ml of Krebs-Ringer phosphate buffer (pH 7.4) containing 5% glucose with slices (wet weight 750 mg) of the cornea and the iris-ciliary body. The incubation was stopped by adding ethanol to the medium.

Extraction and purification of the metabolites

The procedure is outlined in Fig. 3. In short, the precipitate obtained after addition of ethanol was filtered and washed twice with ethanol. The filtrate and the washings were combined and dried by evaporation. The residue was partitioned between ethyl acetate and H_2O. The ethyl acetate fraction was washed with 2N HCl, 5% Na_2CO_3 and H_2O, successively, dried with Na_2SO_4, and evaporated. The residue was chromatographed on a thin layer plate.

In experiments using radioactive dexamethasone, the residue was mixed with authentic dexamethasone and 9α-

fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione and then chromatographed.

Thin layer chromatography (TLC)

TLC plate, 250 mm in thickness, was prepared using Kiezel gel GF 254 (Merck Co., Ltd.).

Solvent systems used were: System A, chloroform/acetone 7:3(V/V); System B, isooctane/isopropylether/isopropylalcohol/acetic acid 2:2:2:1(V/V). Radioactive metabolites on the TLC plate were located by scanning the plate with an Aloka Model TMR-1B thin layer chromatogram scanner. Non-radioactive steroids were detected by inspecting the plate under UV light.

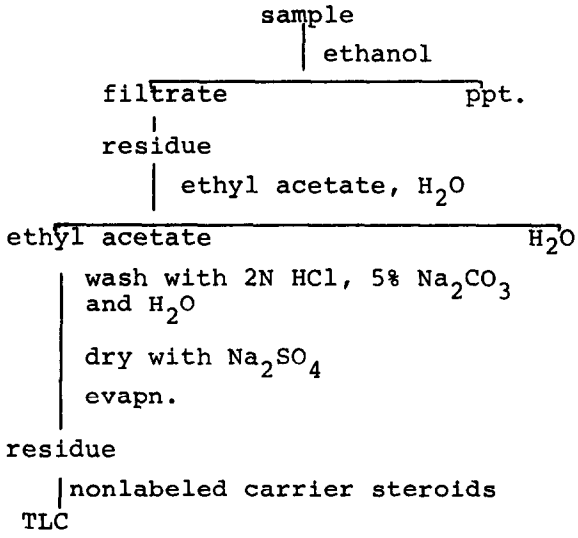

Fig. 3 The outline of analytical method of the metabolites in samples.

Measurement of radioactivity

Radioactive materials were placed in a counting vial, and dissolved in 0.5 ml of methanol. Radioactive counting was performed in the Packard liquid scintillation spectrometer, Model 3002, in a counting vial after adding 10ml of scintillation fluid consisting of 4 g of 2,5-diphenyl oxazol and 100 mg 1,4-bis-2-(5-phenyl oxazolyl) benzene added to 1 liter of toluene. Efficiency of measuring ^3H was 30%.

RESULTS

A) Metabolism of ^{3}H-dexamethasone in vivo

After instilling ^{3}H-dexamethasone into the eyes, the contents of radioactivity in the aqueous humor, the ocular tissues and the serum assessed by measuring the radioactives in aliquots of their ethanol extracts were 1.1%, 10.0% and 7.5% of the administered ^{3}H-dexamethasone, respectively. The radioactive metabolites obtained from these origins were mostly unconjugated, because, after partition between ethyl acetate and water, most of the radioactivity was contained in the ethyl acetate fraction (aqueous humor,98.5%; ocular tissues,93.0%; serum,88.0%).

The metabolites in the ethyl acetate fractions were chromatographed on thin layer plate in system A. The radioscans are shown in Fig. 4. The metabolites in the aqueous humor (Fig. 4A) showed two radioactive peaks, corresponding to the positions of 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione and dexamethasone, respectively. The former peak contained 41.2% and the latter peak 48.7% of radioactivity. The radioactive materials in these peaks were extracted with methanol-acetone 1:1(V/V), separately. The material in the former peak was mixed with nonradioactive 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione and that in the latter peak with nonradioactive dexamethasone, and they were recrystallized from acetone-n-hexane separately to show their radioactive homogeneities

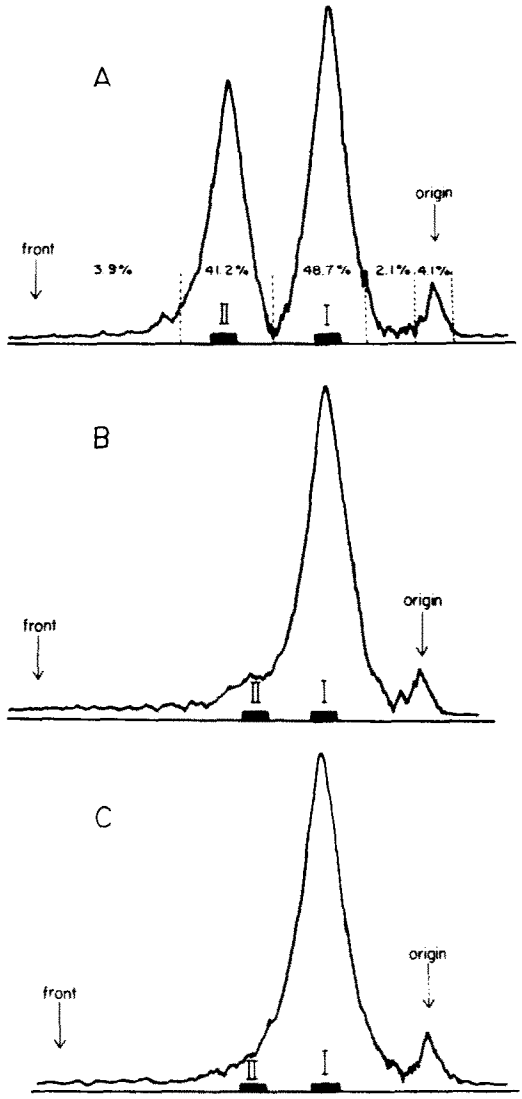

Fig. 4 Thin layer radioscans of the metabolites in A) aqueous humor, B) eye ball tissues, and C) serum after ^{3}H-dexamethasone was instilled into eyes. The marks I and II indicate dexamethasone and 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3, 17-dione added as unlabeled carrier steroids, respectively. Solvent system: ether/acetone 7:3(V/V).

(Table 1). The formation of radioactive 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione was further confirmed by oxidizing it with CrO_3 to give a metabolite with the same mobility on a thin layer plate developed in system A as the product obtained by oxidation of authentic 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione with CrO_3.

Table 1 Recrystallization of the ^3H-metabolites in fractions I and II of the thin layer chromatogram (Fig. 4A) with nonradioactive dexamethasone and 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione, respectively.

	metabolite in fraction (I)	metabolite in fraction (II)
crystallization	specific activity cpm/mg	specific activity cpm/mg
1	180 X 10^3	38.5 X 10^3
2	151 X 10^3	28.7 X 10^3
3	152 X 10^3	28.6 X 10^3

Solvent used was acetone-n-hexane.

The ethyl acetate extract from the ocular tissue or that from the serum showed only one radioactive peak corresponding to the position of dexamethasone (Figs. 4B and 4C).

B) Metabolism of dexamethasone disodium phosphate in vivo

After dexamethasone disodium phosphate solution (Decadron) was instilled into the eye in a similar manner as described above, the aqueous humor was collected and extracted with ethanol. The extract was partitioned between

ethyl acetate and H_2O. The metabolites in the ethyl acetate and H_2O fractions were analyzed separately by thin layer chromatography. Dexamethasone disodium phosphate was not contained in the H_2O fraction, while free dexamethasone and a small amount of 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione were contained in the ethyl acetate extract (Fig. 5).

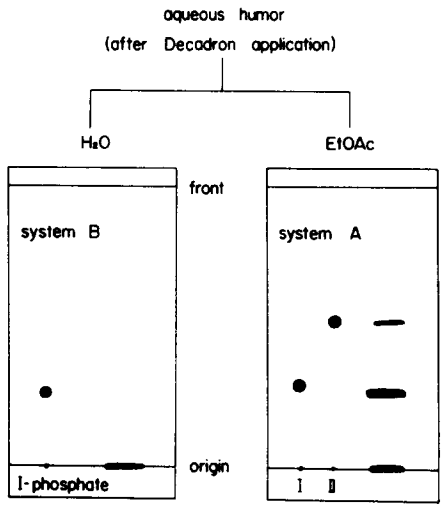

Fig. 5 Analysis of aqueous humor after Decadron solution (5 mg/ml) was instilled into eyes. Aqueous humor was extracted with ethanol. The extract was partitioned between ethyl acetate and H_2O. The metabolites in the ethyl acetate and H_2O fractions were chromatographed.
Solvent systems used were system A for ethyl acetate fraction and system B for H_2O fraction.
I, dexamethasone; I-phosphate, dexamethasone disodium phosphate; II, 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione.

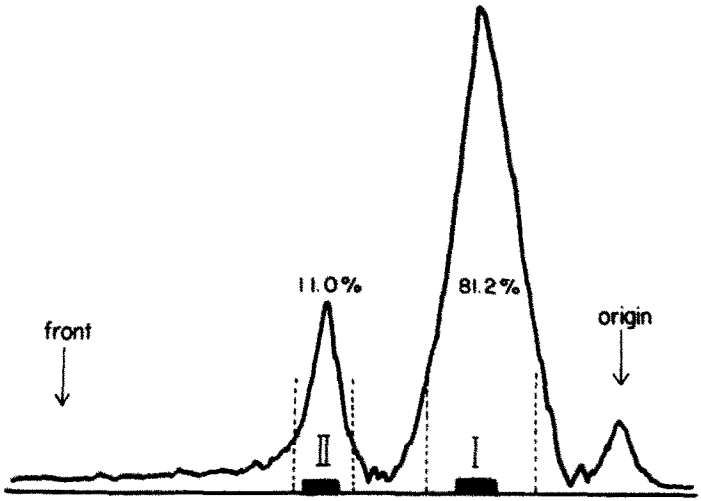

Fig. 6 Thin layer radioscan of the extracted metabolites after ^{3}H-dexamethasone (5 µCi) was incubated at 37 in the air for 1 hr. with slices (wet weight 750 mg) of iris-ciliary body in Krebs—Ringer phosphate buffer containing 5% glucose.
See Fig. 3 regards the solvent system used and carrier steroids added.

C) Metabolism of ^{3}H-dexamethasone in vivo

After instilling ^{3}H-dexamethasone into the eyes, radioactive 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3, 17-dione was found in the aqueous humor. Therefore, slices of the cornea and the iris ciliary body were incubated with ^{3}H-dexamethasone to study the presence of an enzyme which cleaves the side chain of dexamethasone in these tissues. The metabolites extracted with ethyl acetate were analyzed by thin layer chromatography. On the radioscan, a peak corresponding to the position of dexamethasone and a small peak corresponding to the position of 9α-fluoro-11β-hydroxy-16α-

methyl-1,4-androstadiene-3,17-dione were detected (Fig. 6). Therefore, dexamethasone side chain cleavage activity was present in those tissues.

DISCUSSION

In the present experiment, 1.1% of the ^{3}H-dexamethasone which had been instilled into a rabbit's eye was transfered into the anterior chamber. This fractional part of the transfer is comparable to those reported by other workers (5,6).

9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3, 17-dione which was found in the aqueous humor as a major metabolite of dexamethasone and which was formed by incubating dexamethasone with the anterior ocular tissue, had a similar mobility on a thin layer plate as ametabolite of dexamethasone reported by Yamauchi et al. (2). Therefore, it might be possible that they obtained the same metabolite as the one identified in this report.

English et al.(7) analyzed the urine collected for 4 days after oral administration of ^{3}H-dexamethasone to rat and found as the metabolites unchanged dexamethasone, 6β-hydroxy-dexamethasone and a 20-reduced product of dexamethasone. In the present experiment, after analysis of the ocular tissues and the serum of a rabbit which had been instilled ^{3}H-dexamethasone into the eyes, only unchanged dexamethasone was identified and neither the metabolites

reported by English et al.(7) nor 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione were detected.

When dexamethasone disodium phosphate as collyria was instilled into rabbit, free dexamethasone and a metabolite, 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione, were detected in the aqueous humor. The result that dexamethasone disodium phosphate was easily hydrolyzed during its transfer into the aqeuous humor is in accordance with the report of Krupin et al.(1) who found free ^{3}H-dexamethasone in the aqeuous humor after instilling ^{3}H-dexamethasone disodium phosphate into rabbit's eyes, although they did not find the side chain cleavage product.

The reason why the so-called steroid glaucoma arises by administration of corticosteroid is not clear (8,9,10, 11), although several reports on the metabolism of corticosteroid in ocular tissue have been published (12,13,14,15). Shevalev and Lipovetskaya (16) reported that the intraocular pressure was increased by long term administration of testosterone in rats. In the present experiment, it has been proved that dexamethasone disodium phosphate is metabolized into 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione in rabbit's eyes. Since the metabolite has a similar structure as testosterone, the influence of 9α-fluoro-11β-hydroxy-16α-methyl-1,4-androstadiene-3,17-dione on the intraocular pressure is to be studied in future.

ACKNOWLEDGEMENTS

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17. The following trivial names have been used in the text:

Betamethasone, 9α-fluoro-11β,17α,21-trihydroxy-16β-methyl-1,4-pregnadiene-3,20-dione.
Dexamethasone, 9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione.
Fluorometholone, 9α-fluoro-11β,17α-dihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione.

6β-Hydroxy-dexamethasone,
9α-fluoro-6β,11β,17α,21-tetrahydroxy-
16α-methyl-1,4-pregnadiene-3,20-dione