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Assay methodology for prednisolone, prednisolone acetate and prednisolone sodium phosphate in rabbit aqueous humor and ocular physiological solutions

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ABSTRACT

Prednisolone, prednisolone acetate and prednisolone sodium phosphate are glucocorticoids used for ocular, anti-inflammatory therapy. A reversed-phase high-performance liquid chromatographic assay using ultraviolet detection has been developed that affords baseline resolution of the above analytes in balanced salt solutions and rabbit aqueous humor. The drugs can be quantified at 0.025–0.05 μg/ml in the above matrices; 6α-methylprednisolone is used as the internal standard. Both esters of prednisolone are vulnerable to chemical and enzymatic hydrolysis giving prednisolone. Analysis of aqueous humor samples shows prednisolone acetate penetrating/metabolizing primarily to prednisolone; prednisolone sodium phosphate penetrates the cornea giving the ester and alcohol.

INTRODUCTION

Prednisolone sodium phosphate (Pred-P) and prednisolone acetate (Pred-A) are glucocorticoids (Fig. 1) commercially available for ocular, anti-inflammatory therapy. Pred-P is administered in solution at 0.125 or 1.0% (Inflamase Mild® 1/8% or Inflamase Forte® 1%). Pred-A is delivered as a suspension at 0.125 or 1.0% (Pred Mild® or Pred Forte®).

Early *in vivo* penetration studies with the radiolabeled esters in the rabbit [1] suggested Pred-A was superior in its ability to cross the epithelial barrier and gain access to the corneal stroma and aqueous humor. A later study [2], however, showed comparable penetration of the two esters into the cornea and aqueous humor. This study dealt with tritiated Pred-P and Pred-A, although the actual materials quantified in the tissues and fluids were not known. Yamauchi *et al.* [3] showed by thin-layer chromatography in a penetration study with Pred-A that only prednisolone was present in the aqueous humor and cornea after a topical administration of 0.1% suspension of Pred-A. Additional animal studies concerned with the ocular metabolism of Pred-A and Pred-P have not been found in the literature. Recently, a human study [4] indicated that topically administered 0.5% Pred-P (Predsol®, Glaxo) penetrated, metabolizing completely to prednisolone. The bioanalytical methodology involved isotope dilution gas chromatogra-

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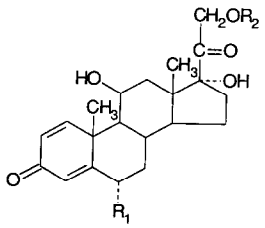

R_1 = -H, R_2 = -H — **Prednisolone**

= $-P(ONa)_2$ — **Prednisolone Sodium Phosphate**

= $-CCH_3$ — **Prednisolone Acetate**

R_1 = $-CH_3$, R_2 = -H — **6α-Methyl Prednisolone** (internal standard)

Fig. 1. Structures of prednisolone and analogues.

phy (GC)–negative-ion chemical ionization mass spectrometry (MS).

Several bioassays in the literature describe methodology for the quantification of systemic concentrations of prednisolone, Pred-A, prednisone and other metabolites [5,6]. These procedures require extraction and concentration of the analytes prior to chromatography. The chromatography involves normal-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) absorbance detection [5–7], GC–chemical ionization MS for human plasma [8], HPLC–MS and isotope dilution MS for serum [9] and radioimmunoassay for plasma [10]. A specific method is not available to quantify Pred-P directly in biological fluids.

The ambiguity of the literature data concerning the metabolism and penetration of Pred-P and Pred-A through the cornea strongly suggests the need for a specific, cold assay that can quantify Pred-A, Pred-P and prednisolone in ocular biological fluids. The available assays do not measure the three analytes in aqueous humor directly. A specific reversed-phase HPLC assay using a UV detector has been developed at 0.025–0.05 μg/ml detection sensitivity. The samples are prepared for HPLC analysis without extraction and are stabilized with arsenic acid at ambient temperature. 6α-Methylprednisolone is used as the internal standard.

EXPERIMENTAL

Materials

Pred-A (1,4-pregnadione-3,20-dione-11β,17α,21-triol 21-acetate) and Pred-P [11β,17,21-trihydroxypregna-1,4-dione-3,20-dione-21-(dihydrogenphosphate) disodium salt] were obtained from Roussel (Englewood Cliffs, NJ, U.S.A.); prednisolone [(11β)-11,17,21-trihydroxypregna-1,4-diene-3,20-dione], prednisone (17,21-dihydroxypregna-1,4-diene-3,11,20-trione) and 6α-methylprednisolone (6α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione) were purchased from Sigma (St. Louis, MO, U.S.A.). The drugs were used as standards and the latter as an internal standard. A balanced salt solution (BSS) from IOLAB Corporation (Claremont, CA, U.S.A.) and a glutathione, bicarbonated ringer solution (GBR) [11] were electrolytic solutions, physiologically similar to fluids in the eye. Arsenic acid was obtained from Sigma and was used to inhibit the enzymatic hydrolysis of Pred-A and Pred-P in biological fluids. The water used was deionized and continuously distilled. Isopropanol was ChromAR HPLC grade from Mallinckrodt (St. Louis, MO, U.S.A.) and was used in the mobile phase. All chemicals were reagent grade and were used as received from the vendors.

Corneas were obtained from adult albino rabbits of both sexes, weighing approximately 3 kg. The sacrificed animals were supplied by El Monte Rabbit Company (El Monte, CA, U.S.A.) and the eyes were excised from the rabbits within 2 h. Tissue viability was determined by percentage hydration of the cornea.

Instrumentation

The chromatographic instrumentation involves a Perkin Elmer LC pump 250, a Perkin Elmer ISS-100 autosampler, a 783A programmable Applied Biosystems absorbance detector and an SP 4270 integrator.

Two columns with the same stationary phase but with different particle and column sizes were used from Dychrom (Sunnyvale, CA, U.S.A.). A Chemcosorb 5-ODS-H (5 μm, 150 mm $\times$ 4.0 mm I.D.) was used for Pred-P and prednisolone and a Chemcosorb 3-ODS-H (3 μm, 75.0 mm $\times$ 4.6 mm I.D.) was used for Pred-A and prednisolone.

HPLC conditions

Chromatography. Mobile phase (1 l) was prepared by mixing 250 ml of isopropanol with 2 ml H_3PO_4 and diluting with water to 900 ml. The pH of the solvent was adjusted to 3.0 with 1.0 *M* NaOH and then diluted to 1 l with water. The solvent was filtered and degassed under vacuum through a Rainin Nylon 0.45 μm filter. The flow-rate was 1.0 ml/min and the column was kept at ambient temperature (22 $\pm$ 2°C).

Detection. The UV absorbance maximum for prednisolone, Pred-A and Pred-P was about 242 nm (ε° 15 000). The detector was set at 245 nm (rise time

1.0), and the range was at 0.01 a.u.f.s. for *in vitro* penetration studies and at 0.002 a.u.f.s. for *in vivo* penetration studies.

The injection volume was 10 μl; the run time was 10 min for the short column and 20 min for the long column. The integrator was set at 16 mV full scale.

Calibration standards

Calibration curves for in vitro studies. Stock solutions of Pred-A were prepared by mixing 7.4 mg of material with 10 ml of methanol in a volumetric flask giving 740 μg/ml; the stock solution was diluted further with water to 74.0 μg/ml in a volumetric flask. These stock solutions were prepared routinely with each new study. The standards were prepared by mixing appropriate volumes of the stock solutions with BSS to achieve concentrations 0.74, 1.85, 3.7, 7.4, 18.5, 37.0 and 74.0 μg/ml.

The stock solutions for prednisolone were prepared as described for Pred-A at 888.0 μg/ml. The standards were mixed with BSS at 0.888, 2.22, 4.44, 8.88 and 17.76 μg/ml.

Stock solutions of Pred-P were prepared with water to give 12.0 mg per 10 ml in a volumetric flask; the stock solution was diluted further with water to 120 μg/ml. The standards were prepared as described above to give concentrations of 1.2, 3.0, 6.0, 12.0, 30, 60 and 120 μg/ml.

Calibration curves for in vivo studies. Stock solutions for Pred-P, Pred-A and prednisolone were prepared as described for the *in vitro* studies at concentrations of 10.1, 10.2 and 10.76 μg/ml, respectively. The calibration standards were prepared with BSS in the ranges 0.050–1.01, 0.050–1.02 and 0.054–1.076 μg/ml, respectively.

Sample preparation for HPLC analysis

A sample aliquot of 50 μl was transferred to the autosampler vial and mixed with 25 μl of 0.5% arsenic acid and 25 μl of internal standard. The vial was capped and the contents were mixed by vortex. The internal standard stock solution was prepared at 3.0 mg per 10 ml methanol; the stock was diluted with water to 30 μg/ml and again to 3.0 μg/ml. The 30 μg/ml stock solution was used for *in vitro* samples with the range of the detector set at 0.01 a.u.f.s.; the 3.0 μg/ml solution was used for *in vivo* samples with the detector set at 0.002 a.u.f.s.

Quality controls

An aliquot (40 or 80 μl) of a drug stock solution was mixed with 0.5 ml of rabbit aqueous humor. The reservoir was individualized into 50-μl aliquots that were stored in capped, conical vials. The vials were kept frozen at −70°C until analyzed by HPLC.

Incubation studies with Pred-A and Pred-P in BSS, aqueous humor and corneal supernatant

The cornea was excised from the rabbit eye and kept in BSS until it was homogenized. The tissue was minced with scissors and manually homogenized in a tissue-grinder (Wheaton) with 1 ml of BSS. The fluid was centrifuged for 15 min at 2000 *g*. The supernatant from different tubes were pooled and kept on wet ice until used.

The incubations involved mixing a 300-μl aliquot of BSS, aqueous humor or corneal supernatant with 25 μl of Pred-A suspension at 0.5% or 25 μl of Pred-P solution at 0.5%. The incubates were shaken in a 37°C water bath over 4 h open to the atmosphere. Aliquots of 50 μl were removed at appropriate times and frozen on dry ice.

In vivo penetration studies

Inflamase Forte® (1%) or Pred Forte® (1%) was topically administered to the right eye of New Zealand White (NZW) rabbits (Irish Farms, Norco, CA, U.S.A). One drop (25 μl) was administered via a pipettor every 5 min upto 30 min. The contra lateral eye served as a control being dosed with saline. Animals were sacrificed (lethal intravenous injection of sodium pentabarbitol) at 30 min, 1 h, 2, 3, 4, 6, and 22 h. Control animals were dosed with saline in both eyes and sacrificed at 3–4 h post-administration. Prior to taking samples, the cornea and conjunctival surfaces were rinsed with saline to remove any residual dose. The aqueous humor samples were removed by paracentesis, using a tuberculin syringe and a 27-gauge needle. The samples were frozen immediately at −70°C (dry ice) and maintained at this temperature.

All animals involved in the *in vivo* experiments were treated in accordance with USDA guidelines and the Resolution of the Use of Animals in Research as put forth by the Association for Research in Vision and Ophthalmology.

RESULTS AND DISCUSSION

Most assays for prednisolone and/or Pred-A use organic extraction (*e.g.*, ethyl acetate, dichloromethane–ethyl ether) procedures of 1 ml or more of plasma, serum or urine to isolate and concentrate the drugs for final analysis [5,6,10]. Other procedures use solid-state support systems to isolate and concentrate the analytes [5,7]. Both procedures will isolate prednisolone from serum at greater than 65% recovery. The extraction procedures will also liberate protein-bound prednisolone [12] which can vary from 90 to 50% with concentration (0.05 to 0.5 μg/ml, respectively) and species [13]. A comparison of liquid–liquid extraction (dichloromethane–diethyl ether, 1:10, v/v) with solid-phase extraction (Bond Elut C_{18}, 500 mg) by Prasad *et al.* [5] demonstrates 70 and 90% extraction recovery of Pred-A and prednisolone from Swine plasma using liquid–liquid extraction *versus* 80 and 90% by the latter procedure. The aqueous humor assay for pred-

nisolone and its esters is a direct procedure where the sample is mixed with arsenic acid and internal standard, and injected into the HPLC column. Extraction of the sample is not considered because of the aqueous humor sample size and the fact that Pred-P does not extract into an organic phase; protein binding is considered minimal.

Traditional methods of protein denaturization and liberation of bound drugs in plasma involve mixing acetonitrile, methanol, perchloric acid, trichloric acetic acid, etc. with a plasma aliquot. Aqueous humor samples containing prednisolone were mixed with acetonitrile (1:2, v/v), with 4.0 *M* perchloric acid (1:1, v/v) or with water (controls, 1:1 and 1:2, v/v) and the prednisolone peak response was compared by HPLC–UV. Addition of acetonitrile or perchloric acid to aqueous humor did not produce a protein precipitate and the peak responses for prednisolone were not significantly different from controls. The results suggest the presence of very little protein in aqueous humor and/or very little protein binding. Other investigators [13] have observed plasma protein binding by adding the labeled prednisolone to plasma via dialysis at 37°C. Binding varied between 90 and 50% depending on drug concentration and plasma species. In a similar ex-

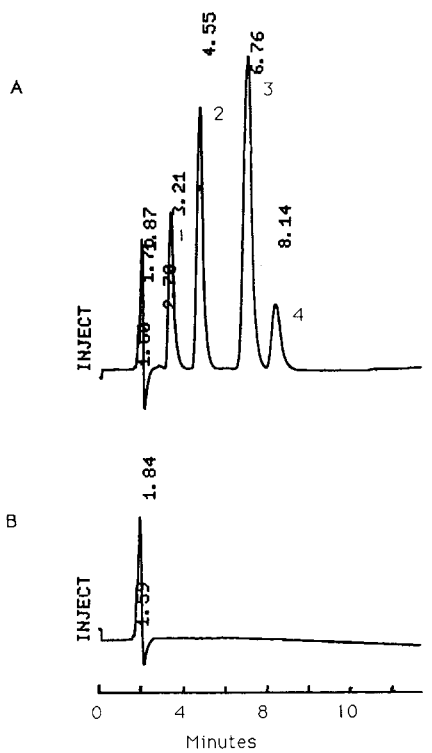

Fig. 2. (A) Chromatogram of a balanced salt solution containing Pred-P (1), prednisolone (2), 6α-methylprednisolone (3) (internal standard) and Pred-A (4); (B) chromatogram of control balance salt solution. The column used was a Chemcosorb 3-ODS-H (5 μm, 150 mm × 4.6 mm I.D.).

periment prednisolone was added to aqueous humor and to water (controls) and incubated at 37°C for 1 and 2 h; the HPLC–UV peak responses of prednisolone were compared with those of the two matrices. There were no significant differences in these two matrices.

The chromatographic methodology used to separate Pred-A, Pred-P and prednisolone is reversed-phase HPLC using a C_{18} stationary phase; most of the HPLC methodology in the literature describes normal-phase chromatography. The analytes can be baseline-separated with a Chemcosorb C_{18} column as shown in Fig. 2. With aqueous humor, the 3-ODS-H column is more appropriate for prednisolone and Pred-A for shorter run times (Fig. 3) and the 5-ODS-H column for prednisolone and Pred-P for resolution from background peaks (Fig. 4). The aqueous humor gives a large background peak near the solvent front and it can interfere with Pred-P using the 3-ODS-H column; this interference is not a problem for Pred-A using this column. The 3-ODS-H column also allows for shorter analysis time of Pred-A (10 min).

The minimum assay sensitivity for all three analytes is about 50 ng/ml with reproducibility under 10% and a 10-μl injection volume. The analytes can be detected at lower levels, especially with larger injection volumes; endogenous background peaks do not interfere. This is limited by the availability of aqueous humor which varies from collection to collection with an average volume of 0.1 ml. A micromethod is described in the literature [14] for the extraction and concentration of 0.1 ml of serum for the analysis of cortisol. The procedure gives a two-fold concentration in dichloromethane (10 μl) which is injected into a normal-phase HPLC system; the technique, however, has nominal advantages with these ophthalmic assays (*e.g.*, Pred-P cannot be isolated by organic extraction).

The calibration standards are prepared with an isotonic, physiological balanced salt solution [15] as a substitute for aqueous humor. The test drugs are stable to chemical hydrolysis of the ester moiety in BSS at room temperature for at least 5 h (Table I). The calibration concentration ranges prepared are dependent on the application of the assay. For *in vitro* corneal penetration studies, the range is 1.0 μg/ml and up; for *in vivo*, the range varies from 0.05 to 1.0 μg/ml. The assays are linear with coefficients of variation of $< 10\%$ (Table II).

Pred-P appears primarily vulnerable to enzymatic hydrolysis but not chemical degradation, giving prednisolone. The enzymatic hydrolysis of Pred-P at 37°C in corneal supernatant correlates with the formation of prednisolone (Fig. 5). Incubation of Pred-P in BSS, in aqueous humor and in corneal supernatant (0.04% solution, w/v) at 37°C generates prednisolone, primarily in the latter matrix. The hydrolysis of Pred-P in the supernatant can be inhibited with the addition of arsenic acid (Fig. 6). Sodium fluoride, which is used to inhibit esterase in plasma [5] did not slow the formation of prednisolone from Pred-P in the corneal supernatant (Fig. 6). The chemical hydrolysis of Pred-P has been tested in 0.1 ml of sodium phosphate at pH 6.0, 7.0 and 8.0 at 37°C. HPLC analysis did not show any significant changes with time in the formation of prednisolone over 24 h.

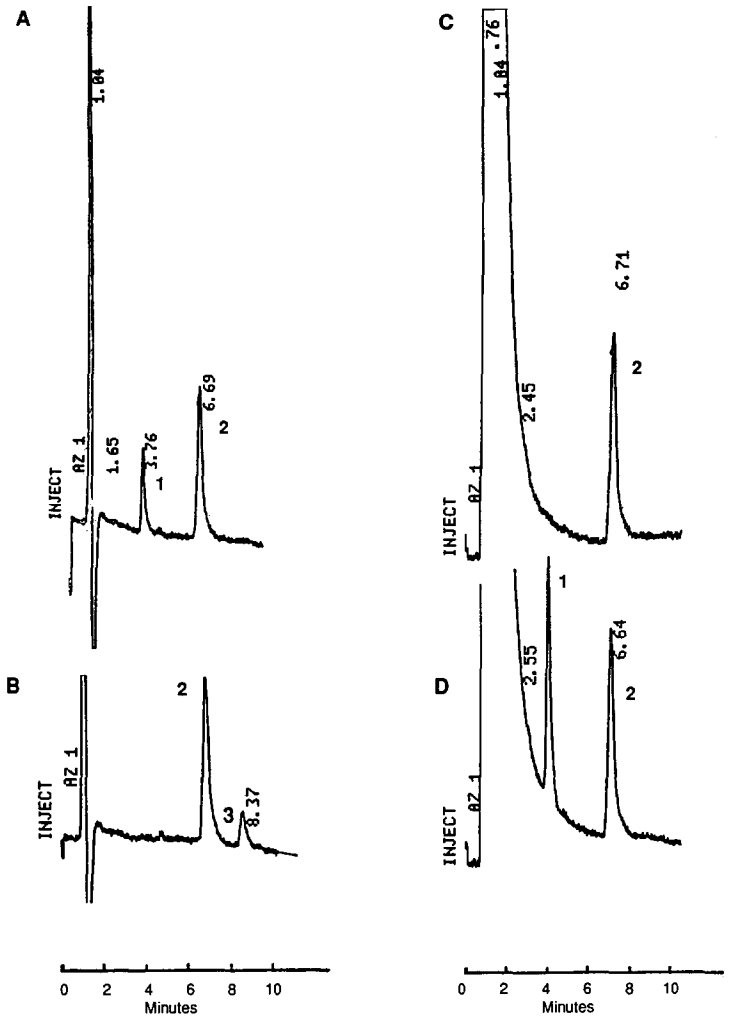

Fig. 3. (A) Chromatogram of a BSS standard containing prednisolone (1) (0.455 μg/ml) and the internal standard (2); (B) chromatogram of a BSS standard containing internal standard and Pred-A (0.501 μg/ml) (3); (C) chromatogram of an aqueous humor sample from the left eye of a rabbit dosed with saline and containing internal standard; (D) chromatogram of an aqueous humor sample from the right eye dosed with 1% Pred-A and containing prednisolone and internal standard. The column used was a Chemcosorb 3-ODS-H (75 mm × 4.6 mm I.D.).

The mechanism of hydrolysis of Pred-A seems to be a combination of enzymatic and base catalysis. Chemical hydrolysis appears to become significant above pH 7.0 (Table III). A comparative incubation of Pred-P in BSS, in aqueous humor and in corneal supernatant (0.04% suspension, w/v) demonstrates the enzymatic contribution. Unlike Pred-P, the inhibitory effects of arsenic acid on

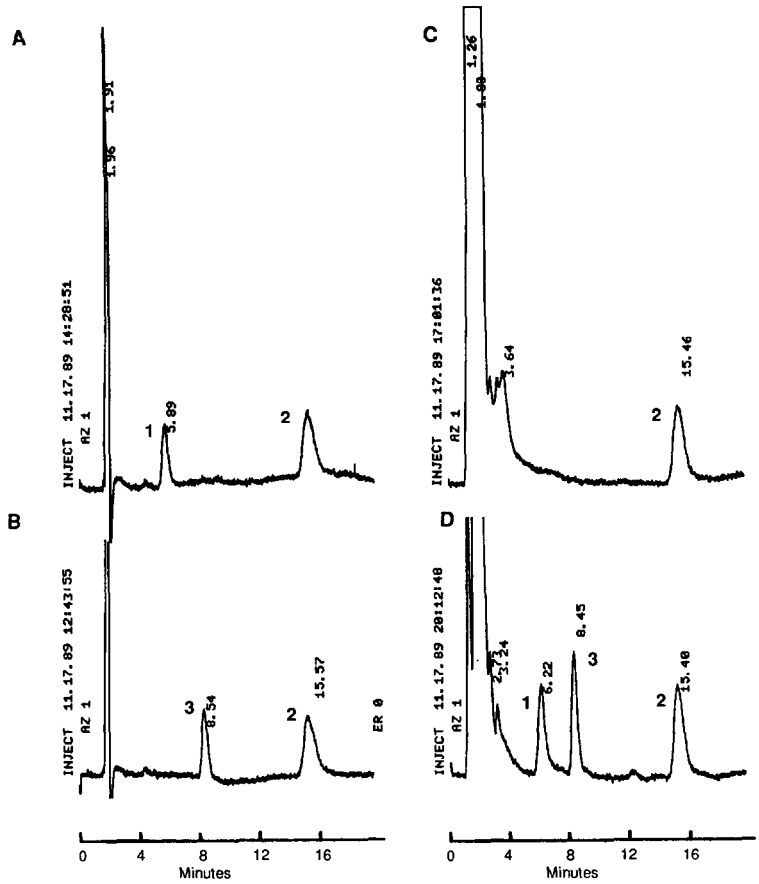

Fig. 4. (A) Chromatogram of a BSS standard containing Pred-P (1) (1.00 μg/ml) and the internal standard (2); (B) chromatogram of a BSS standard containing prednisolone (3) (0.455 μg/ml) and internal standard; (C) chromatogram of an aqueous humor sample from the left eye of a rabbit dosed with saline and containing internal standard; (D) chromatogram of an aqueous humor sample from the right eye (3-h collection) dosed with Pred-P and containing prednisolone (1), Pred-P (3) and internal standard. The column used was a Chemcosorb 5-ODS-H (150 mm × 4.6 mm I.D.).

the enzymatic hydrolysis of Pred-A in the corneal supernatant appears minimal (Fig. 7).

The accuracy and inter-day precision of the assays for the quantification of the three analytes in rabbit aqueous humor are shown in Table IV. The quality controls were individualized in capped vials and stored at −70°C. The controls were analyzed over a period of five days.

The stability of Pred-A has been observed in swine plasma at room temperature [5] showing "reasonable stability" upto 3 h with "quantitative conversion" to prednisolone within 24 h. In aqueous humor at room temperature Pred-A

TABLE I

STABILITY OF PRED-P AND PRED-A IN BSS AT ROOM TEMPERATURE

Concentrations of Pred-P and Pred-A were 11.1 and 10.4 μg/ml, respectively.

Time (h)	Percentage remaining	
	Pred-P	Pred-A
0.00	100	100
1.45	97.1	107.2
2.92	96.0	99.7
4.37	95.6	99.7
5.84	96.7	98.5

TABLE II

LINEAR REGRESSION PARAMETERS FOR PREDNISOLONE, PRED-A, AND PRED-P IN ELECTROLYTE SOLUTIONS

Drug	Calibration range (μg/ml)	Mean coefficient of variation[a] (%)	Linear regression		
			r^2	Slope	y-Intercept
Prednisolone	0.054–1.076	4.79 ± 2.17	0.9993	1.00	0.0137
	0.888–17.7	5.62 ± 3.97	0.9994	0.1177	0.0152
Pred-A	0.0501–1.01	6.86 ± 1.62	0.9985	0.266	0.0199
	0.74–37.0	4.07 ± 4.62	0.9957	0.1187	0.1002
Pred-P	0.050–1.009	5.01 ± 1.99	0.9988	1.064	0.0403
	1.4–14.0	6.31 ± 2.32	0.9969	0.1045	0.1417

[a] Reproducibility at four concentrations with five replicates at each concentration.

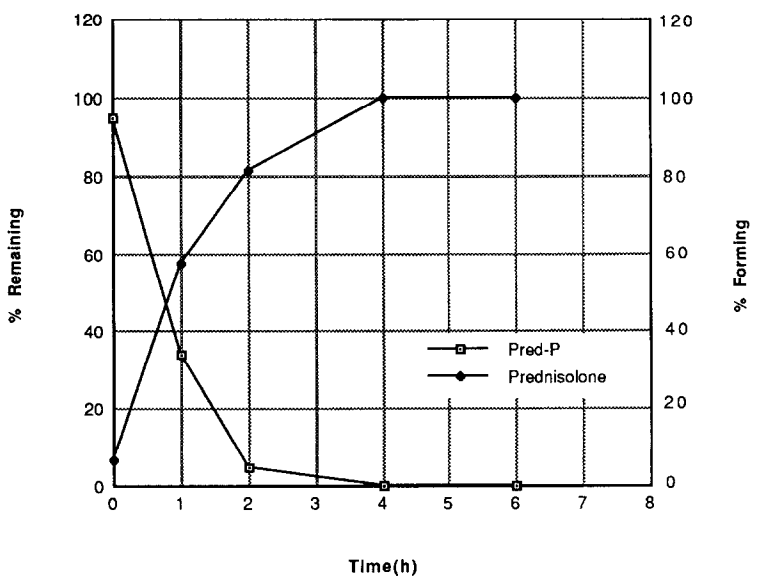

Fig. 5. Enzymatic hydrolysis of Pred-P to prednisolone in corneal supernatant at 37°C.

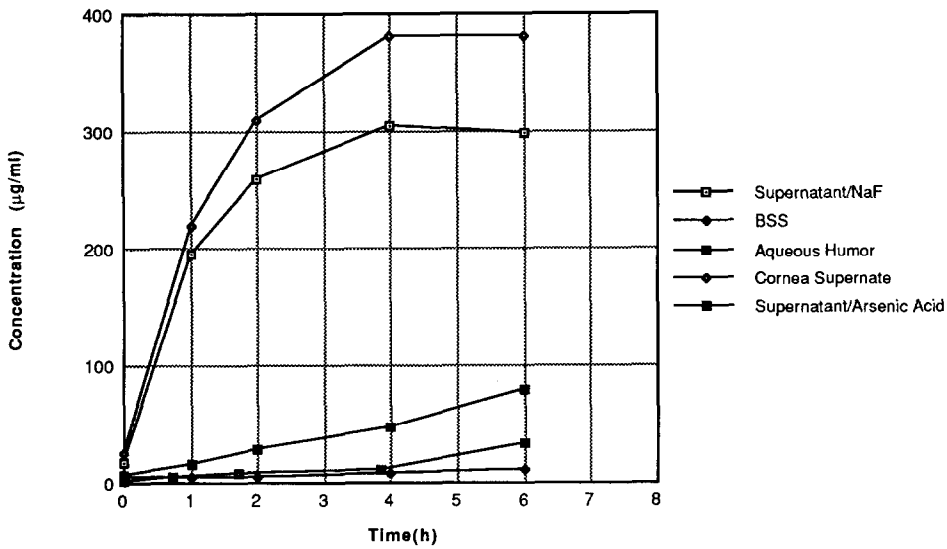

Fig. 6. Incubations of Pred-P in BSS, rabbit aqueous humor and cornea supernatant. The plot represents the formation of prednisolone in various matrices.

shows reasonable stability upto 4 h at 10 µg/ml with arsenic acid and degrades, thereafter, to below 95% of initial concentration. Pred-P (0.04%, w/v) is stable in aqueous humor in the presence of arsenic acid upto 6.3 h. In GBR, Pred-P shows stability upto 5 h while Pred-A is stable upto 2.65 h and continues to degrade to below 90.0% at 5 h. The pH of GBR (contains a carbonated buffer) rises from 7.4 to over 8.0 with time explaining the accelerated degradation rate of Pred-A in GBR (Fig. 7).

TABLE III

CHEMICAL HYDROLYSIS OF PRED-A TO PREDNISOLONE IN 0.1 *M* SODIUM-PHOSPHATE BUFFERS AT pH 6.08, 7.05 AND 8.36

Pred-A concentration is 200 µg/ml (suspension); shaken in a water bath at 37°C.

Time (h)	Prednisolone formed (µg/ml)		
	pH 6.08	pH 7.05	pH 8.36
0.00	0.1477	0.5247	2.142
1.00	0.2022	0.6619	4.485
2.75	0.1787	0.9402	7.893
4.25	0.2539	0.9958	10.29
5.75	0.2068	1.313	16.568

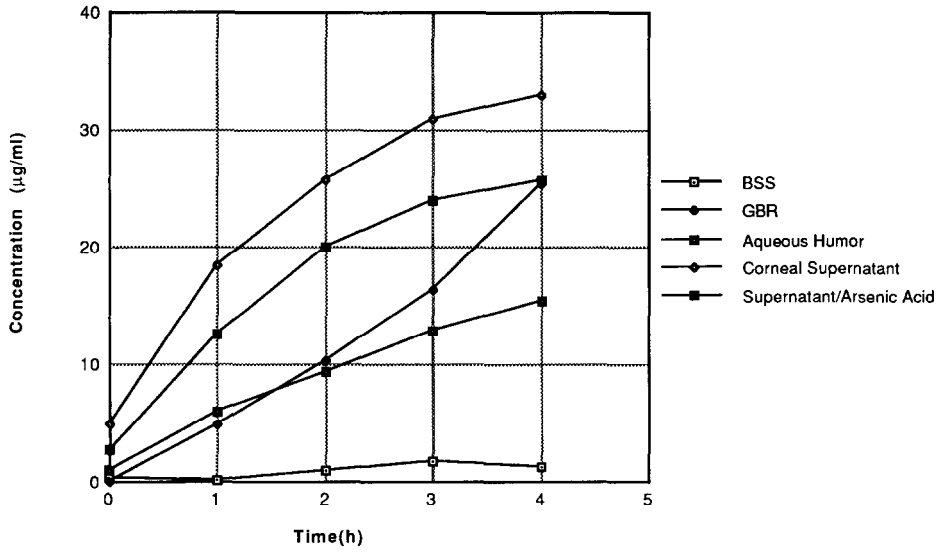

Fig. 7. Incubations of Pred-A in BSS, GBR, rabbit aqueous humor and corneal supernatant at 37°C. The plot represents the formation of prednisolone in various matrices.

In vivo and *in vitro* corneal penetration studies (NZW rabbit) with Pred-A demonstrate almost complete hydrolysis of the ester to its metabolite prednisolone (Fig. 3). This observation has been previously reported by Yamauchi *et al.* [3] using radiolabeled drug, by Richman and Tang-Liu [16] using HPLC–UV and by McGhee *et al.* [4] in humans using GC–negative-ion chemical-ionization MS.

Current *in vitro* and *in vivo* animal studies [17,18] (rabbit) with Pred-P detect the presence of both Pred-P and prednisolone in the anterior chamber (Fig. 4). The human study by McGhee *et al.* [4] is the only corneal penetration/metabolism

TABLE IV

INTER-DAY REPRODUCIBILITY OF THE RABBIT AQUEOUS HUMOR ASSAY FOR PRED-A, PRED-P AND PREDNISOLONE

Steroid	Expected concentration (µg/ml)	Found concentration (µg/ml)	*n*	Coefficient of variation (%)
Pred-P	0.30	0.348	4	8.31
	0.60	0.619	4	8.59
Pred-A	0.40	0.416	3	3.99
	0.80	0.753	3	6.98
Prednisolone	0.40	0.427	3	9.11
	0.80	0.824	3	6.06

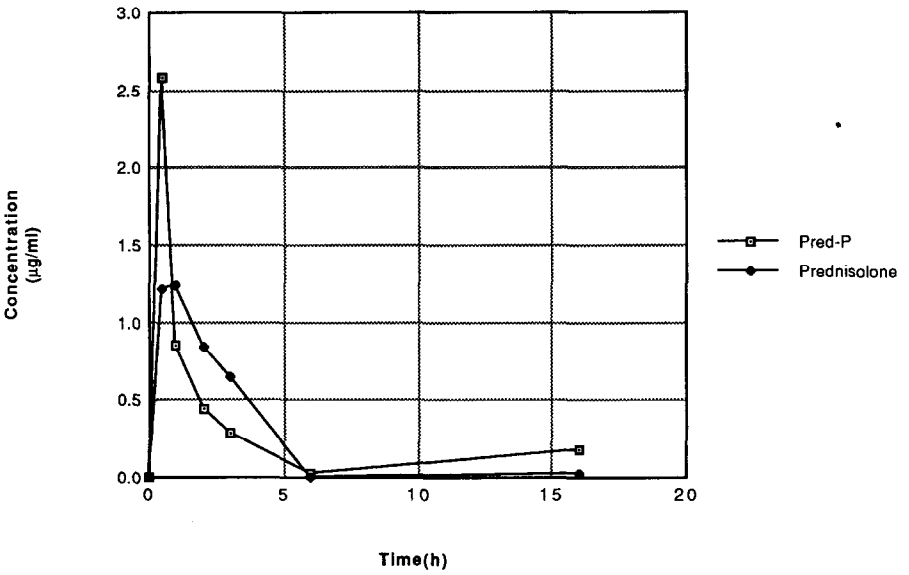

Fig. 8. Concentration profiles of Pred-P and prednisolone in rabbit aqueous humor via Pred-P administration (1%) (each point is an average of two determinations).

work with Pred-P found in the literature using a cold assay and shows Pred-P to be totally metabolized to prednisolone. This is determined indirectly (without controls) by extracting free prednisolone from aqueous humor (from human subjects topically administered with 0.5% Predsol), incubating the aqueous humor

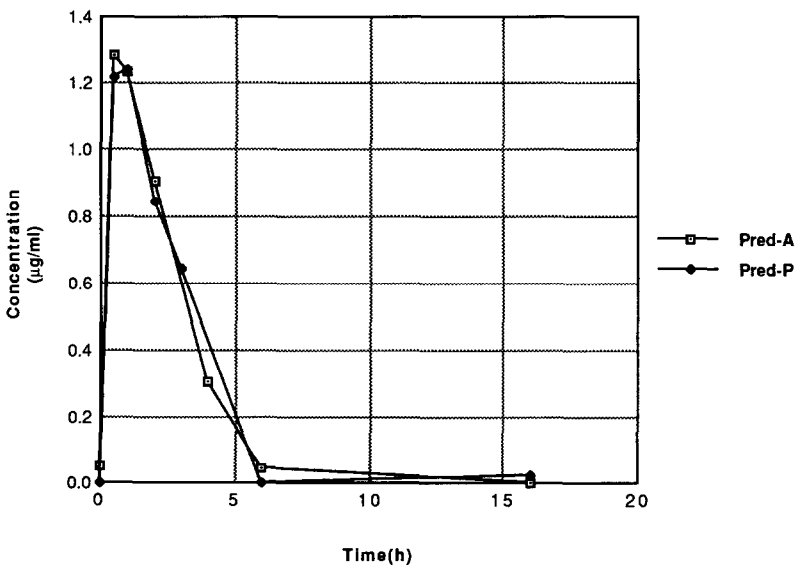

Fig. 9. Concentration profiles of prednisolone aqueous humor via Pred-A and Pred-P administration into the right eye of rabbits (each point is an average of two determinations).

with phosphatase and further extracting any prednisolone that may have formed from Pred-P.

Representative aqueous humor profiles of Pred-P, prednisolone and Pred-A after topical administration of Inflamase Forte® 1% and Pred Forte® 1% are shown in Figs. 8 and 9. Multiple topical application of both preparations were administered to the right eye of each rabbit every 5 min for 0.5 h in order to reach a steady-state concentration of the drugs in the cornea. The plots of prednisolone formed from Pred-P and Pred-A (Fig. 9) overlap with areas under the curve (0–6 h) of 3.75 and 3.58 μg h/ml, respectively.

CONCLUSION

The assay methodology for prednisolone, Pred-A and Pred-P chromatographically baseline-resolves the analytes in rabbit aqueous humor and ocular physiological solutions with direct injection of the samples into the HPLC column. The methodology does not require an extraction procedure for Pred-A or for prednisolone or an indirect enzymatic hydrolysis/extraction procedure for Pred-P to quantify. Both esters are vulnerable to enzymatic and chemical hydrolysis, particularly Pred-A. Arsenic acid has a significant inhibitory effect on the enzymatic hydrolysis of Pred-P and a much less effect on Pred-A.

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