Korean J. Ophthalmol.
Vol. 9:79-83, 1995

Determination of Dexamethasone Sodium Phosphate in the Vitreous by High Performance Liquid Chromatography

Hyung Woo Kwak, M.D. and Donald J. D'Amico, M.D.*

Department of Ophthalmology, School of Medicine, Kyung Hee University , Seoul, Korea,
**Department of Ophthalmology, Harvard Medical School, Boston, MA, U.S.A.*

Dexamethasone sodium phosphate (DSP) is increasingly used in the treatment of ocular inflammatory diseases by systemic, periocular, and recently, intravitreal injection. We have developed a method for the determination of vitreous levels of DSP by reverse phase HPLC. In this method, co-elution of vitreous proteins with DSP is resolved by a prior sample clean-up procedure using Waters Sep-Pak C18 cartridge; the protein is separated and eluted with water while DSP, paraben and prednisone are eluted with methanol. DSP in the resulting sample is then separated by reverse phase HPLC and quantified by UV absorption at 254 nm. The recovery of DSP through the sample clean-up is 68.9 ± 3.0%. DSP quantitation is linear from 0.1 mg to 1.0 mg per 1.0 ml vitreous. This method provides a simple, sensitive and reliable technique for determining the vitreous levels of DSP.

Key words: dexamethasone, dexamethasone sodium phosphate, steroids, vitreous, HPLC (high performance liquid chromotography).

INTRODUCTION

Dexamethasone is a synthetic steroid which is widely used in the treatment of ocular inflammatory diseases. In the treatment of endophthalmitis, the intravitreal injection of steroids has been found beneficial in animal models, and its clinical use (in association with intravitreal antibiotic injections) is under investigation. Experimental models of proliferative vitreoretinopathy have also suggested a role for the intravitreal injection of steroids.

Dexamethasone alone is poorly suited to intravitreal use because its limited solubility in aqueous media would require the injection of potentially-toxic solvent vehicles into the eye.[1] However, dexamethasone sodium phosphate (DSP) has a high degree of solubility and stability in aqueous solution, and commercial preparations of DSP are widely available as aqueous solutions (e.g. Decadron, Merck Sharp and Dohme, Philadelphia, PA is a combination of DSP, methylparaben, propylparaben, creatinine, sodium citrate, sodium hydroxide, and sodium bisulfite). The toxicity and clearance of intraocular DSP has not been determined previously. The intravitreal doses of DSP that have been employed in animal studies are very low (200 - 400 μg); consequently, clearance studies require a very sensitive technique for analysis. In previous studies radioimmunoassay (RIA) has been used.[2,3] However, RIA suffers from poor specificity and reproducibility when analyzing steroid levels in the vitreous. Dexamethasone is routinely determined in biological fluids by reverse phase high performance liquid chromatography (HPLC).[4] DSP has different chemical properties than dexamethasone and by HPLC it is difficult to

Reprint requests to Hyung Woo Kwak, M.D., Department of Ophthalmology, School of Medicine, Kyung Hee University, Hoeki-Dong, Dongdaemun-Ku, Seoul 130-702, Korea.

separate DSP from the other components in biological fluids. We have developed a procedure for the measurement of vitreous levels of DSP. This technique will enable further studies of the vitreous turnover and metabolism of DSP and DSP-containing preparations. In addition, with modification this technique may permit the determination of DSP in other biological fluids.

MATERIALS AND METHODS

Chemicals and reagents

Pure dexamethasone sodium phosphate, a gift from Merck Sharp and Dohme (Philadelphia, PA), was dried at a pressure of 5.0 mm of mercury at 40° C to constant weight before using.[5] Decadron was commercially obtained from Merck Sharp and Dohme. Prednisone, dexamethasone and paraben were purchased from Sigma (St. Louis, MO). Methanol used during the procedures was HPLC grade.

Vitreous sample preparation

Rabbits were sacrificed by an overdose of intravenous ketamine, following the ARVO Resolution on the Use of Animals in Research. Eyes were enucleated, sectioned at the equator, and the vitreous was removed. The vitreous was centrifuged at 13,000 g for ten minutes. Prednisone was added as an internal standard to supernatant collected. The supernatant was then applied to a Sep-Pak C-18 cartridge (Waters Associates, Milford, MA) and eluted as described in results.

Chromatography

Chromatography was performed on a Waters (Milford, MA) 820 system consisting of 2 model 510 pumps, a WISP model 720 autosampler and a model 481 UV detector. Separations were done on a Waters μ Bondapak C18 column run isocratically at 1.0 ml/min. using 50:50 mixture of methanol:water as a solvent. Peaks were identified by comparing them to the retention times of pure standards and quantified by UV absorption peak area at 254 nm. Protein concentration was measured using BCA protein assay reagent (Pierce Chemical Co, Rockford, IL).

DSP concentration, with prednisone as an internal standard, was calculated as follows:

$$\text{DSP } \mu\text{g/ml} = C \times \{RF\,(DSP) \times PA\,(DSP)\} / \{RF\,(\text{Prednisone}) \times PA\,(\text{Prednisone})\}$$

C = final concentration of standard solution
PA= peak area.
RF (DSP), RF (Prednisone) = average response factor for peak area of DSP and prednisone

RESULTS

For this analysis it is necessary to separate DSP from other components in the injectable formulation of the drug Decadron. Fig. 1 shows the optimized separation of DSP, paraben (a preservative in Decadron), dexamethasone and prednisone (used as an internal standard). Peak retention times were found to be reproducible to within 2.0%. Increased methanol in the eluant resulted in shorter retention times. Conversely decreased methanol in the eluant resulted in longer retention times.

The vitreous containing Decadron and prednisone was analyzed by HPLC and the resulting chromatogram demonstrated that the predominant chromatographic interference in the vitreous sample was protein (Fig. 2A). The protein concentration in the vitreous was determined to be 0.82 $\pm$ 0.25 mg/ml. A procedure to separate the protein from the steroids was then developed using Sep-Pak C18

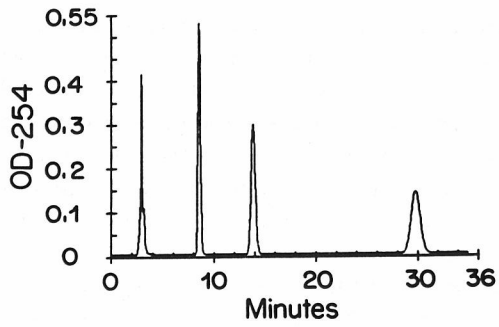

Fig. 1. Optimized separation of the components of Decadron and internal standard. Separation is done using a Waters μ Bondapak C18 column eluted with 1:1 methanol:water at a ml/min. Peaks are DSP at 2.9 min, paraben at 8.5 min, prednisone at 13.8 min and dexamethasone at 29.8 min.

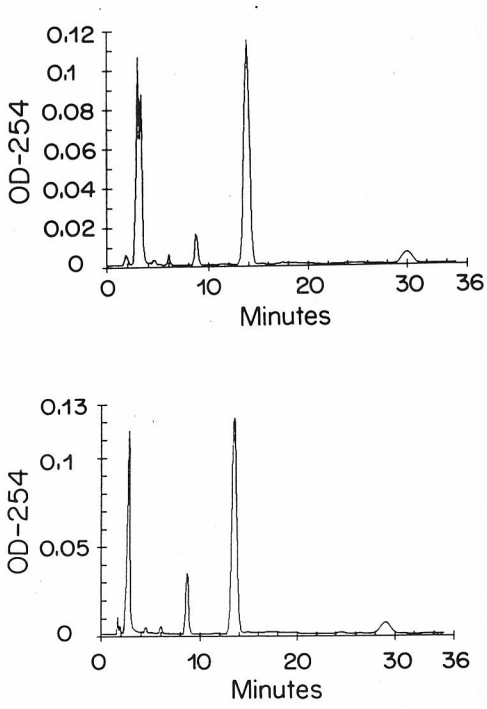

Fig. 2. (A) Chromatogram of the sample before Sep-Pak C18 cartridge clean-up. The chromatogram displays prominent chromatographic interference in the peak area of DSP. (B) Chromatogram of the sample after Sep-Pak C18 cartridge clean-up procedure. The chromatogram displays no interference in the peak area. The peaks of DSP, paraben, and prednisone are visible at 2.8, 8.6, and 13.8 min.

cartridges. After samples were purified with Sep-Pak C18 cartridges we were then able to identify DSP, protein, paraben and prednisone at different steps by a protein assay reagent and spectroscopy. Protein was eluted with water and DSP, paraben and prednisone were eluted within methanol. The percent recoveries are shown in Table 1. The standard sample contained 100 μg DSP, 15 μg. paraben and 100 μg. prednisone in a volume of 0.5 ml. The recovery of DSP through this clean-up was 68.9 $\pm$ 3.0 %. and recovery of the prednisone was 92.4 $\pm$ 1.7 %. For the preparation of the standard curve, the 1.0 ml of the blank vitreous was mixed with DSP (0.1-1.0 mg), prednisone (0.1-1.0 mg) and paraben (0.06-0.3 mg). The mixtures were vortexed for 1 min. and applied to a Sep-Pak C18 cartridge. The methanol eluate was dried by a speed vacuum concentrator (Savant, Farmingdale, NY). Each residue was then resuspended in 1.0 ml of methanol and vortexed. The final sample separation after Sep-Pak treatment is shown in Fig. 2B.

The resulting samples were analyzed by HPLC

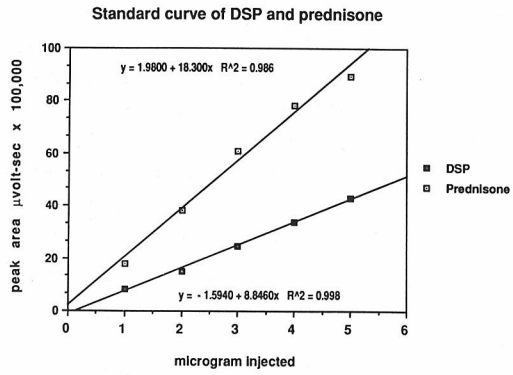

Fig. 3. Standard curve of DSP and prednisone after Sep-Pak C18 cartridge clean-up procedure. Peak area was determined by the Waters model 820 data system. Lines were determined by least-squares regression.

Table 1. Percentage of recoveries from Sep-Pak sample pretreatment*

	% DSP	% Paraben	% Protein	% Prednisone
Sample Application	0.0	0.0	3.7 $\pm$ 2.7	0.0
8.0 ml Water	26.5 $\pm$ 3.8	0.0	79.4 $\pm$ 3.7	0.0
0.5 ml MeOH	1.1 $\pm$ 1.0	0.0	1.6 $\pm$ 2.2	0.0
2.0 ml MeOH	68.9 $\pm$ 3.0	92.4 $\pm$ 1.7	12.7 $\pm$ 2.6	91.9 $\pm$ 3.4

**Prior to the use, the cartridge was prewet with 2.0 ml of methanol followed by 5.0 ml of water. The vitreous was then applied to a Sep-Pak C18 Cartridge. The cartridge was then washed with 8.0 ml of distilled water, 0.5 ml methanol and eluted with 2.0 ml of methanol. During the elution procedure, a sample of each step was collected.*

and the findings may be summarized as follows. In HPLC the region of DSP elution was found to be free from interference by any vitreous component. The standard curves of DSP and prednisone are shown in Fig. 3. Computer analysis of the observed ratio revealed a linear relationship between peaks area and amount injected with a correlation coefficient of 0.998 for DSP, 0.986 for prednisone.

DISCUSSION

Numerous corticosteroids are employed in the treatment of the ocular inflammatory diseases and each steroid may exist as a number of derivatives. Recently, the use of the synthetic steroids in the vitreous has increased. Among them, DSP has the greatest potential because it is highly potent with an activity approximately ten times that of prednisone.[6,7] It is also highly water soluble and available as an aqueous preparation that would avoid the potential problems of ocular toxicity associated with non-aqueous vehicles required to prepare pure dexamethasone solutions. The intraocular toxicity and the clearance of DSP have not been defined and it is not supplied commercially for intraocular use. Dexamethasone and other steroids may be analyzed in biological fluids using RIA,[3,8,9] but the specificity and reproducibility by RIA are low. HPLC methods have greater specificity and precision; however, the analysis of DSP in biological fluids has not been reported. In our study, the DSP displayed a different retention time and chemical properties than dexamethasone. We could not identify DSP in samples without clean-up because of the high interference by vitreous protein, which is caused by similarities in retention times of DSP and protein in HPLC. Recently, a similar sample clean up procedure using a Sep-Pak C18 cartridge for the extraction of urinary steroids has been described.[10] The present study used a Sep-Pak C18 cartridge and a reverse phase HPLC in the purification step to enhance the sensitivity and to correct for the interference due to protein. The results with this sample clean-up procedure confirm that the chromatogram of the DSP has been corrected for the interference from endogenous vitreous components such as protein.

This report has presented a sensitive and reproducible technique for the HPLC analysis of DSP in the vitreous. Although recovery following the sample clean-up procedure is reduced (68.9%), it is highly reproducible. This method will permit evaluation of the pharmacokinetics and bioavailability of DSP in the vitreous following systemic, periocular, and intravitreal injection; this data will be particularly useful in the ongoing development of steroid therapy for a wide variety of ocular disorders. In addition, it is possible that a modification of this procedure may permit the measurement of DSP in other biological fluids.

REFERENCES

1. Maurice, D.M.: *Injection of drugs into the vitreous body. In Symposium on Ocular Therapy, Leopold IH, and Burns RP, editors. New York, John Wiley & Sons, 1976, pp. 59-71.*
2. Hare, L.E., Yeh, K.C., Ditzler, C.A., McMahon, F.G., and Duggan, D.E.: *Bioavailability of dexamethasone: II. Dexamethasone phosphate. Clin. Pharmacol. Ther. 18: 330, 1975.*
3. Graham, R.O. and Peyman, G.A.: *Intravitreal injection of dexamethasone: Treatment of experimentally induced endophthalmitis. Arch. Ophthalmol. 92: 149, 1974.*
4. Lamiable, D., Vistelle, R., Millart, H., Sulmont, V., Fay, R., Caron, J., and Choisy, H.: *High performance liquid chromatographic determination of dexamethasone in human plasma. J. Chromatogr. 378: 486, 1986.*
5. United States Pharmacopeia XXII, National Formulary XVII. Rockville, U.S Pharmacopeial Convention, 1989, pp. 397.
6. Nielsen, R.H.: *The use of dexamethasone in ophthalmologic steroid therapy: A preliminary report. Arch. Ophthalmol. 62: 438, 1959.*
7. Haynes, R.C. and Murad, F.: *Adrenocorticotropic hormone. Goodman and Gilman's the Pharmacological Basis of Therapeutics. 7th edition, Gilman AG, Goodman LS, Rall TW,and Murad F, editors. New York, Macmillan, 1985, pp. 1466.*
8. Leopold, I.H., Kroman, H.S., and Green, H.: *Intraocular penetration of prednisone and prednisolone. Trans. Am. Acad. Ophthalmol. 59: 771, 1955.*
9. Short, C., Keates, R.H., Donovan, E.F., Wyman, M., and Mudick, P.W.: *Ocular penetration studies: I. Topical administration of dexamethasone. Arch. Ophthalmol. 75: 689, 1966.*

10. Shackleton, C.H.L. and Whitney, J.O.: *Use of Sep-Pak cartridges for urinary steroid extraction: Evaluation of the method for use prior to gas chromatographic analysis. Clin. Chem. Acta 107: 231, 1980.*