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Short communication

Determination of dexamethasone in tears by capillary electrophoresis

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Abstract

A selective capillary zone electrophoresis (CZE) microassay was developed for the simultaneous determination of dexamethasone phosphate and its major metabolite, dexamethasone, in tears. The calibration was carried out in the biological matrix with indoprofen as an internal standard which allowed the separation of dexamethasone phosphate and dexamethasone from the tear constituents. The limits of detection and quantification of the assay were 0.5 and 2.0 $\mu g\ ml^{-1}$, respectively. This quantification method is essential for the in vivo determination of dexamethasone concentration–time profiles in tears after application of the antiinflammatory drug.

Keywords: Dexamethasone; Corticosteroids; Steroids

1. Introduction

Corticosteroids are widely used in the treatment of many inflammatory diseases [1–4]. Among the corticosteroids employed in systemic and ocular therapy, dexamethasone has the highest estimated potency [3]. Furthermore, dexamethasone is generally resistant to degradation and is likely to have a relatively long half time in tissues [3,5].

The very small volumes used clinically in ophthalmic treatments (30 to 50 μl) coupled with limited tear volumes (approximately 7 μl in men and rabbits) create great difficulties in developing suitable analytical methods to assess the pharmacokinetics of dexamethasone in tears.

Several authors suggested the use of radioimmunoassay to assess the bioavailability of oral dexamethasone and intravenous dexamethasone esters [6–11], unfortunately, this analytical method suffers from poor specificity and reproducibility when dosing in the tear fluid is required [12].

Specific and precise HPLC methods have been widely used [13–15]. However, without derivatization, they lack sensitivity and therefore cannot be used for accurate determination of concentrations in the $\mu g\ ml^{-1}$ range for sample volumes of 1–2 μl.

More recently, mass spectrometry techniques have been developed for measuring dexamethasone in biological fluids [12,16–18], but the high degree of technical skill and the cost of sophisticated equipment prevent its application on a routine basis.

The present paper describes the development of a new, simple, sensitive and reproducible assay using capillary zone electrophoresis (CZE). This assay allows the selective determination of dexamethasone and dexamethasone phosphate in tears. The main

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advantage of CZE is the small sample volume that is required for an injection. In addition, the procedure does not require any sample derivatization or purification. This method of quantification can be applied to monitor tear concentration–time profiles of topically administered dexamethasone eye drops.

2. Experimental

2.1. Instrumentation

Experiments were carried out on a HP^{3D}CE system (Hewlett-Packard, Wilmington, DE, USA). This system consisted of a capillary electrophoresis unit equipped with a diode array detector (DAD), an autosampler and a high-velocity air-cooled capillary cartridge. The HP^{3D}CE ChemStation software was used for instrument control, data acquisition and data analysis. Hewlett-Packard capillaries with a 50-μm I.D. (375 μm O.D.), 64.5 cm total length (56 cm from inlet to the detector window) were used for all experiments. These capillaries were made of fused-silica and equipped with an extended path-length detection window of 150-μm I.D. ('bubble cell'). New capillaries were flushed for 3 min with 1 *M* NaOH, then 5 min with 0.1 *M* NaOH, and finally 10 min with water. Between each run, capillaries were flushed for 4 min with separation buffer. When analyzing tear samples, capillaries were flushed after the run with 0.1 *M* sodium dodecyl sulphate (1 min), and then 3 min with the separation buffer [19].

2.2. Chemicals

Dexamethasone phosphate and indoprofen (used as internal standard: I.S.) were purchased from Sigma (St. Louis, MO, USA). The separation buffer was made of sodium tetraborate decahydrate from Siegfried (Zofingen, Switzerland). The sample solvent was an isocryoscopic phosphate buffer solution (PBS) which contained Na_2HPO_4 from Siegfried, NaH_2PO_4 from Merck (Dietikon, Switzerland) and NaCl from Fluka (Buchs, Switzerland). Ultrapure water was provided by a Milli-Q RG unit from Millipore (Bedford, MA, USA).

2.3. Electrophoretic conditions

Samples (ca. 24 nl) were injected by pressure (5 kPa for 20 s) and electrophoresis was performed at a constant voltage of 25 kV (388 V cm^{-1}) after a 1 min ramp step to avoid loss of sample at the injection [20]. The capillary was thermostated at 25°C and the detection was performed using a diode array detector (DAD) scanning the wavelengths from 190 nm to 600 nm. Electropherograms were monitored at 242 nm with a bandwidth of 3 nm for the dexamethasone, and at 300 nm with a bandwidth of 2 nm for the internal standard. In order to substract the detector noise, the reference signal was fixed in both cases at 450 nm (bandwidth=80 nm). In all experiments, areas were corrected by their respective migration times.

2.4. Buffer preparation

The 100 m*M* sodium tetraborate buffer used for the separation was prepared by dissolving 19.1 g of sodium tetraborate decahydrate in 500.0 ml of water. The pH was 9.2.

The isocryoscopic phosphate buffer (PBS) used for the samples was prepared by dissolving 0.04 g of NaH_2PO_4, 0.28 g of Na_2HPO_4 and 8.5 g of NaCl in 1000 ml of water.

All the buffer solutions were filtered through a 0.45-μm Nylon syringe filters before use.

2.5. Standard samples preparation

Known solutions of dexamethasone phosphate in PBS ranging from 2 μg ml^{-1} up to 100 μg ml^{-1}, with a fixed concentration of 50 μg ml^{-1} for the I.S., were used for the validation of the method. Before injection, 20 μl of this standard solution was added into the micro-vial and then centrifuged at 700 *g* for 5 min (Centaur 2 MSE, Zivy, Oberwil, Switzerland).

2.6. Tear samples preparation

A micro-vial (Hewlett-Packard, Waldbronn, Germany) was filled with 18 μl of PBS containing 50 μg ml^{-1} of I.S.. Then, 2 μl of tears were collected with a micro-capillary (Microcaps Drummond, Thomas Scientific, Swedesboro, NJ, USA), and

blown under a gentle nitrogen flow into the micro-vial. The vial was finally centrifuged for 5 min at 700 *g* before injection.

3. Results and discussion

3.1. Calibration of dexamethasone phosphate

Because of the small tear volume involved in this assay, the I.S. was essential to correct for the evaporation that may have occurred in the sample vial and for the injection volume variation [19]. As shown in Fig. 1, I.S. is completely separated from dexamethasone phosphate with migration times of 12.26 min and 15.46 min, respectively.

The limits of detection and quantification were respectively 0.5 $\mu g\,ml^{-1}$ (signal-to-noise ratio 3:1) and 2 $\mu g\,ml^{-1}$ (signal-to-noise ratio 10:1). The linearity of the response was demonstrated from 2 to 100 $\mu g\,ml^{-1}$. The correlation coefficient (r) was 0.9997. The regression equation gave a slope of 0.014 (standard deviation <0.001) and an intercept of -0.003 ± 0.008. The intercept was not statistically different from zero. Repeatability of the method has been tested on 6 replicate injections of standards. Migration times and normalized areas showed a coefficient of variation of 0.6% and 2.1%, respectively.

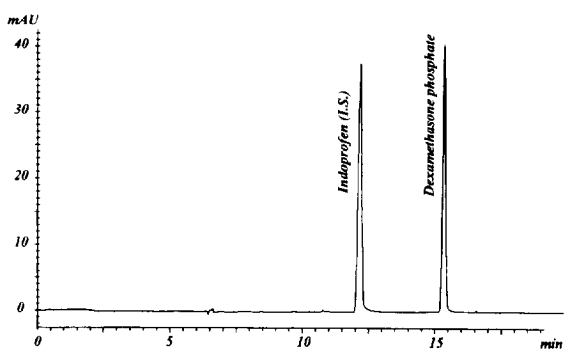

Fig. 1. Separation of a sample containing indoprofen (I.S.) (50 $\mu g\,ml^{-1}$) and dexamethasone phosphate (100 $\mu g\,ml^{-1}$) in PBS. Experimental conditions: buffer = 100 m*M* borate (pH 9.2); injection = 100 kPa s (approx. 23.8 nl); $E = 388\ V\,cm^{-1}$ (1 min voltage ramp); $T = 25°C$; detection wavelength = 242 nm; bandwith = 3 nm.

3.2. Chemical shift from dexamethasone phosphate into dexamethasone

Esterases are known to be present in the cornea as well as in tears [3,21]. Fig. 2 shows a shift from dexamethasone phosphate into dexamethasone, due most probably to the presence of these enzymes. Due to the loss of the negatively charged phosphate group, dexamethasone can easily be detected and isolated from dexamethasone phosphate and tear constituents. Dexamethasone has been identified by its UV spectrum, and showed a migration time of 7.05 min.

As shown in Fig. 2, dexamethasone migrates just after the EOF, defined by the baseline irregularity [22]. It is well known that borate can form weak complexes with molecules containing diol moieties (e.g., sugar derivatives, flavonoids), resulting in a low anionic mobility behavior [23–26]. Dexamethasone may have a similar behavior, which could explain its anionic mobility.

During the shift, the F ratio between the dexamethasone and the dexamethasone phosphate was constant over the range of concentrations in which the response is linear (Table 1). The quantitation of total dexamethasone was then possible by using the following equation:

$$\text{area}_{\text{total dexamethasone}} = \text{area}_{\text{dexamethasone phosphate}} + \frac{\text{area}_{\text{dexamethasone}}}{F_{\text{average}}}$$

where F_{average} corresponds to the mean value of the

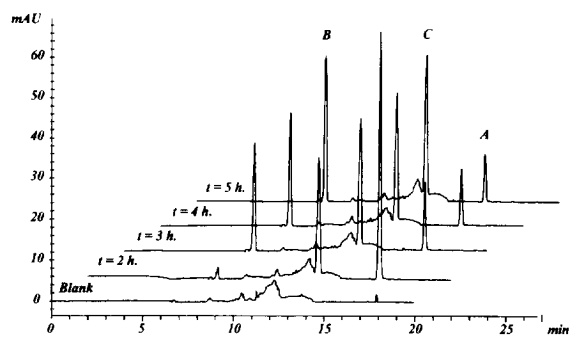

Fig. 2. Chemical shift from dexamethasone phosphate (A) to dexamethasone (B), as function of time, in a spiked tear sample (100 $\mu g\,ml^{-1}$ of dexamethasone phosphate and 50 $\mu g\,ml^{-1}$ of internal standard (C)). For experimental conditions, see Fig. 1.

Table 1
Determination of the F ratio for the quantitation of total dexamethasone

Concentration ($\mu g\ ml^{-1}$)	$Area_{dexamethasone\ phosphate}$ (AU)[a]	$Area_{dexamethasone}$ (AU)[a]	F ratio[b]
2	0.028	0.039	1.392
10	0.135	0.194	1.437
25	0.353	0.488	1.382
50	0.678	0.961	1.417
75	1.031	1.420	1.377
100	1.386	1.905	1.374
$F_{average}$			1.396

[a] AU = arbitrary units defined as: peak areas divided by their respective migration time.
[b] F ratio = $area_{dexamethasone}/area_{dexamethasone\ phosphate}$.

F ratio determined in the concentration range from 2 to 100 $\mu g\ ml^{-1}$ (Table 1).

After 24 h, no trace of dexamethasone phosphate was detected any more. To avoid interference with tears components, the detection of indoprofen was determined at 300 nm (Fig. 3).

3.3. Calibration of total dexamethasone in the biological matrix

Since dexamethasone phosphate loses its phosphate moiety in the presence of the lachrymal esterases, the calibration curve was realized using total dexamethasone as reference signal. The limits of detection and quantification were respectively 0.5 $\mu g\ ml^{-1}$ (signal-to-noise ratio 3:1) and 2 $\mu g\ ml^{-1}$ (signal-to-noise ratio 10:1). The response of the assay was linear from 2 to 100 $\mu g\ ml^{-1}$ and the correlation coefficient (r) for the calibration curve

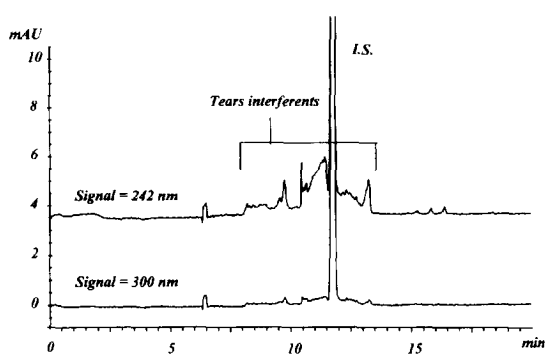

Fig. 3. Detection of indoprofen (I.S.) at different wavelengths to avoid interferences with tears components. For experimental conditions, see Fig. 1.

was 0.9994. The regression equation gave a slope of 0.019 (standard deviation <0.001) and an intercept of 0.005 ± 0.008. The intercept was not statistically different from zero; this means that no matrix effect from tears is found in this calibration.

The method is sensitive enough for studying dexamethasone concentration in tears following administration of ocular preparations. Furthermore, no matrix effect can be observed in the range of concentrations enabling the use of the CZE mode. If lower sensitivity was required, MEKC would be a useful tool, since a better specificity for corticosteroids has been demonstrated [27,28].

4. Conclusions

A selective method was developed for the determination of dexamethasone and dexamethasone phosphate in tears. Because of the extremely small volumes available for the analysis, CZE proved to be an excellent method, and sufficiently sensitive to determine dexamethasone concentration in biological fluids such as tears. The method was found to be cost effective, rapid and reliable in comparison with other analytical techniques.

After a full validation, this method will be a useful tool for studying tear concentration of dexamethasone following administration of ocular preparations containing this drug and for investigating bioequivalence of formulations. This method could also be used to study ocular pharmacokinetic parameters of other active compounds, since no matrix effect was

noted between the neutral and negative charged species in tears.

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