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# Assay of the synthetic estrogen fosfestrol in pharmaceutical formulations using capillary electrophoresis

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**Abstract**

This study reports – for the first time – a capillary electrophoretic method for the determination of fosfestrol, a synthetic estrogen used in the treatment of metastatic prostate cancer. The effects of the carrier ion concentration, injected volume and applied voltage were studied and optimized. A 10 mM sodium tetraborate solution was selected as the carrier electrolyte solution, while the sample was injected hydrodynamically by applying a 20 mmHg vacuum for 1 s. The driving voltage was 30 kV and the absorbance of the analyte (peak height) was monitored at 240 nm. Under the above-mentioned conditions, the migration time of fosfestrol was 6.6 min. Linearity was achieved in the analyte range 3–150 mg $L^{-1}$ with the detection limit being 1 mg $L^{-1}$. The proposed method is adequately precise ($s_r$ = 2.8% at 100 mg $L^{-1}$ fosfestrol, $n$ = 10) without the use of an internal standard and was applied to the determination of fosfestrol in a pharmaceutical formulation. The results obtained by the proposed method were in good agreement with those derived from the USP reference method.


*Keywords:* Capillary electrophoresis; Fosfestrol; Pharmaceuticals

## 1. Introduction

Fosfestrol (diethylstilbestrol (DES) diphosphate (Fig. 1) is an inactive synthetic estrogen (female hormone), used as a pro-drug of the active DES. It has been proved to be highly efficient in the treatment of hormone-refractory prostatic carcinoma [1]. The advantage of using fosfestrol instead of DES is that the pro-drug is not active in the serum and, thus, has fewer side effects. It is activated in the target cells, where it reaches higher concentrations than the levels achieved if DES is administrated. Pharmacokinetic studies have shown that fosfestrol metabolites have a short half-life, supporting its suitability for long-term infusions in the clinic. It relieves the patients' urinary symptoms, such as poor flow, by shrinking the tumor [2].

The importance for monitoring and assuring the quality of fosfestrol-containing formulations can be easily concluded by its expected side effects, which are basically the same as those of estrogens: nausea and vomiting, weight gain, edema and gynecomastia. However, the most serious, life threatening, complications that may occur are thromboembolic events [3].

So far, the reported fosfestrol assays are based on high performance liquid chromatography (HPLC) [4–9], batch [10,11] and automated spectrophotometry using flow injection analysis [12–14]. Capillary electrophoresis (CE) is a relatively "young" technique [15,16] gaining continuously growing attention and acceptance among scientists due to its unique features and advantages over traditional liquid chromatographic separation techniques. To the best of our knowledge no capillary electrophoresis-based methods have been developed for the determination of fosfestrol.

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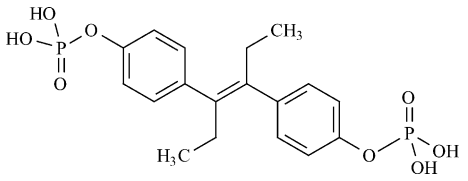

Fig. 1. Chemical structure of fosfestrol.

This work reports the first capillary electrophoresis-based assay for the synthetic estrogen fosfestrol. The analyte was suspected to be suitable for CE-analysis as its phosphoric ester groups can be ionized effectively at basic pH and it has a well-defined absorbance maximum at 240 nm. The effects of running buffer concentration, sample injection volume and applied separation voltage were studied and optimized. Key validation parameters including linearity, precision and accuracy of the CE method were evaluated and the method was applied to the analysis of a fosfestrol-containing pharmaceutical formulation.

## 2. Experimental

### 2.1. Instrumentation

A 270A-HT Applied Biosystems capillary electrophoresis instrument was used throughout this work. Analysis/separation was performed in 70 cm × 50 μm i.d. (effective length 50 cm) fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). Electropherograms were recorded and displayed on a PC by means of a multifunction I/O card (PCI 524E, National Instruments, TX, USA) through a data acquisition application developed in LabVIEW 5.1 (National Instruments); the same program also performed peak evaluation. An Orion EA940 pH-meter was employed for the pH measurements with absolute accuracy limits at the pH measurements being defined by NIST buffers.

### 2.2. Chemicals

All solutions were prepared in pre-filtered (through 0.45 μm membrane filters) doubly de-ionized water (Millipore water purification system, MilliQ® gradient, Millipore S.A., Bedford, USA). After preparation, the solutions were filtered again to avoid capillary clogging from non-dissolved particles.

Sodium tetraborate, $Na_2B_4O_7$, was used as carrier–buffer solution and was prepared by dissolving the appropriate amount of $Na_2B_4O_7{\cdot}10H_2O$ (Merck, Darmstadt, Germany) in water.

Fosfestrol tetrasodium reference material (quality control no. 101386 K, batch no. 9K23002, assay 99,6%) was kindly donated by Asta Medica Inc. (Frankfurt, Germany). Standard stock solutions were prepared daily by dissolving the appropriate amount of the analyte in 100 ml of doubly de-ionized water. Working solutions were prepared by dilution immediately before use. All fosfestrol solutions were kept protected from the light.

### 2.3. Capillary electrophoretic procedure

When a capillary was used for the first time, it was preconditioned with 0.1 mol $L^{-1}$ NaOH for 20 min, then with water for 10 min and with air for 5 min, finally followed by flushing with electrolyte solution for 20 min. The capillary was conditioned daily by washing it with ultrapure water (5 min), followed by freshly prepared 0.1 mol $L^{-1}$ NaOH (5 min), ultra pure water (5 min) and fresh buffer (20 min). In order to optimize the migration time and peak shape reproducibility, the capillary was flushed after each completed run with 0.1 mol $L^{-1}$ NaOH (1 min), ultrapure water (2 min) and fresh buffer (2 min). The daily closing down procedure consisted of rinsing the capillary with distilled water for 10 min, followed by flushing with air for 5 min. The capillary was always stored dry overnight.

The sample was introduced to the capillary hydrodynamically by applying a 20 mmHg vacuum for 1 s. The applied voltage was 30 kV and fosfestrol was detected spectrophotometrically at 240 nm through the optical window that was made 50 cm from the injection point. All experiments were carried out at room temperature.

### 2.4. Determination of fosfestrol in pharmaceutical samples

Twenty tablets of the fosfestrol-containing formulation were divided into two equal batches. Each batch was weighed and grounded in a mortar to a fine powder. An appropriate amount of each batch was dissolved in doubly de-ionized water (to a concentration of ca. 100 mg fosfestrol $L^{-1}$), filtered and then analyzed using the CE procedure described above.

### 2.5. Determination of fosfestrol by the official method

A quantity of the ground fosfestrol-containing tablets corresponding to ca. 100 mg of the analyte was dissolved in 25 ml of ethanol. After the addition of 5 ml of 1 mol $L^{-1}$ NaOH solution the mixture was diluted to a final volume of 250 ml with doubly de-ionized water. Five milliliters of the resulting solution was further diluted to 100 ml with doubly de-ionized water. The absorbance of the final solution was measured spectrophotometrically at 240 nm [17].

## 3. Results and discussion

### 3.1. Study of CE variables

During method development several parameters were studied and optimized. These parameters were run buffer concentration, pH, sample injection volume and applied

Table 1
Effect of the concentration of $Na_2B_4O_7$ on the peak height and the migration time of fosfestrol (100 mg $L^{-1}$)

| $c(Na_2B_4O_7)$ (mM) | $H$[a] ($s_r$[b](%)) | $t_m$ (min[c]) ($s_r$[b](%)) |
|---|---|---|
| 5.0 | 88023 (5.2) | 4.2 (1.7) |
| 10.0 | 90998 (3.0) | 6.6 (0.8) |
| 20.0 | 90017 (3.2) | 9.0 (1.2) |
| 50.0 | – | >21 |

[a] $H$ = peak height in arbitrary units.
[b] $s_r$ = relative standard deviation.
[c] $t_m$ = migration time.

voltage. All experiments were carried out using a 100 mg $L^{-1}$ standard fosfestrol solution. It should be noted that – for simplicity reasons – quantitation was evaluated by height measurements, as preliminary studies showed equal precision and accuracy compared to peak area. The results correspond to arbitrary units.

For selecting a buffer solution as a carrier for the CE-analysis, the separation was optimized with respect to the analysis time and sensitivity of the determination. A 10 mM $Na_2B_4O_7$ solution gave a fosfestrol peak at about 6.5 min, a migration time that was considered satisfactory for selecting this solution as the carrier. The effect of the carrier solution concentration was studied in a range 5–50 mM. The sample was injected hydrodynamically by applying a vacuum of 20 mmHg for 1 s. The applied voltage was 30 kV. No significant changes in the signal (ca. 10%) were observed for different concentrations of borate solutions. On the other hand, the migration times varied, resulting in larger times as the concentration increased. In general, when the concentration of the borate buffer is low, the electroosmotic flow is high and it has an apparent appearance in the electropherogram. The migration times and the relative standard deviation of the corresponding peaks and migration times are shown in Table 1. A borate solution of 10 mM was selected instead of 5 mM, due to better precision. The pH of the solution was 8.95. At this pH the migration time and peak shape were considered satisfactory and no further studies were made.

The injection volume was studied by varying the time interval during which a vacuum of 20 mmHg was applied. Time variation in the range 0.2–2.0 s resulted in – as expected – higher peaks. Better precision was achieved using an injection time of 1 s, which was finally selected.

It is known that the applied voltage has a significant effect in the migration time of the analyte and in fact, increased voltages generally result in shorter migration times. In general, when the applied voltage is increased, the column efficiency and the selectivity reach a maximum and then decrease as the voltage is further increased, due to the inefficient heat dissipation within the capillary and shorter migration times of the solutes. Its effect was studied by applying voltages of 10, 20 and 30 kV. The respective migration times of fosfestrol were > 20, 14.1 and 6.5 min. The driving voltage of 30 kV was selected in terms of shorter analysis time.

### 3.2. Linearity studies

Under the preferred conditions ($c$ $Na_2B_4O_7$ = 10 mM, $\lambda$ = 240 nm, $V$ = 30 kV, $t_{inj}$ = 1 s (20 mmHg)), a linear calibration graph was obtained in the range 3–150 mg $L^{-1}$ fosfestrol. The achieved linearity covers the 50–150% range of the expected analyte concentration in the samples (100 mg fosfestrol per tablet), which is a typical demand in pharmaceutical analysis. The calibration graph was described by the equation,

$$H = (-1148 \pm 1237) + (977 \pm 16)\gamma\,(\text{fosfestrol})$$

where $H$ is the peak height measured by the detector (in arbitrary units), and $\gamma$ (fosfestrol) is the mass concentration of the analyte with a relative standard deviation of $s_r$ = 2.8% (at 100 mg $L^{-1}$ fosfestrol, $n$ = 10), a correlation coefficient of $r$ = 0.9989, a 3$\sigma$ limit of detection of 1 mg $L^{-1}$ and a limit of quantitation of 3 mg $L^{-1}$. All standards were determined by five replicate injections ($n$ = 5).

### 3.3. Precision studies

The repeatability and intermediate precision of the method were checked with within-day and inter-day assays. In the first case a 100 mg $L^{-1}$ fosfestrol solution was repeatedly measured ($n$ = 12) at five different times during the same day. The relative standard deviation of the gathered results ranged between 2 and 3%. The inter-day precision was evaluated by comparing the slopes of the regression lines of the resulting calibration curves prepared on five different days over a one-week period. The average linear coefficient was $r$ = 0.9988 and the relative standard deviation of the slopes of the five curves was 6.2%.

### 3.4. Accuracy studies

The accuracy of the developed assay was proved by analyzing synthetic – validation – samples prepared by the addition of known amounts of fosfestrol to a placebo matrix. The placebo was a mixture of the excipients found in the real samples, namely: Maize starch 84% (w/w), Colloidal silicon dioxide 4.3% (w/w), Magnesium stearate 5% (w/w) and Talc 6.7% (w/w). Accurately weighed amounts of the analyte – in the range 50–150% – were mixed with ca. 200 mg of the placebo mixture, diluted to 200 ml, sonicated for 10 min and analyzed by the proposed method after filtration through 0.45 μm disposable syringe filters. The experimental results of Table 2 verified the accuracy of the CE method, as the mean percent recovery was 99.6%.

### 3.5. Determination of fosfestrol in pharmaceutical samples

The proposed method was applied to the analysis of a commercially available formulation containing fosfestrol (Honvan, Asta Medica Inc.). An electropherogram of Honvan

Table 2
Accuracy studies; determination of Fosfestrol in synthetic samples

| Sample no. | Placebo added (mg) | Fosfestrol added (mg/200 ml) | Fosfestrol added (mg $L^{-1}$) | Recovery[a] (%) |
|---|---|---|---|---|
| 1 | 200 | 10.2 | 51.0 | 99.1 |
| 2 | 200 | 9.9 | 49.5 | 99.3 |
| 3 | 200 | 20.5 | 102.5 | 100.8 |
| 4 | 200 | 20.4 | 102.0 | 101.1 |
| 5 | 200 | 30.2 | 151.0 | 98.7 |
| 6 | 200 | 29.7 | 148.5 | 98.5 |

[a] Mean of three results.

Table 3
Determination of fosfestrol in a pharmaceutical formulation

| Sample | Fosfestrol added (mg $L^{-1}$) | Fosfestrol found[a] (mg $L^{-1}$) | $R$[b] (%) | Official method[c] | $e_r$[d] (%) |
|---|---|---|---|---|---|
| S1 | – | 118.3[e] | – | 120.8 | −2.07 |
| | 20 | 19.8 | 99.0 | – | |
| | 50 | 51.4 | 102.8 | – | |
| S2 | – | 119.6[e] | 99.7 | 121.1 | −1.24 |
| | 20 | 20.7 | 103.5 | – | |
| | 50 | 51.6 | 103.2 | – | |

[a] Mean of five results.
[b] Percent average recovery.
[c] Fosfestrol tetrasodium found by the USP method in mg per tablet.
[d] Relative error.
[e] Fosfestrol tetrasodium found in mg per tablet.

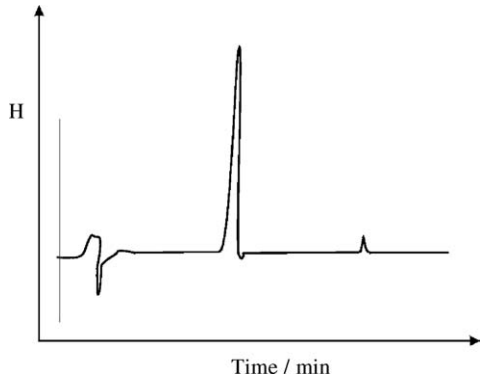


Fig. 2. Electropherogram of Honvan sample solution.

sample solution is given in Fig. 2. The results are summarized in Table 3. The calculated average recoveries were satisfactory in all cases, ranging between 98.9 and 103.4%. The accuracy of the proposed method was investigated by comparing the results obtained by the proposed method with those found by application of the official USP method [17]. These comparisons are also shown in Table 3, and verified the accuracy of the proposed method as the relative errors ranged between –1.24 and –2.07%.

## 4. Conclusions

This work describes the first capillary electrophoretic method for the determination of a synthetic estrogen, fosfestrol. The assay is simple, does not require toxic organic solvents, has a short analysis time and produces minimum waste. The key performance characteristics such as linearity, accuracy, precision and limit of detection were satisfactory and it was successfully applied to the analysis of a commercially available pharmaceutical formulation. Compared to the USP photometric assay it offers the possibility of determining the analyte – after suitable pre-treatment – in biological fluids, based on the separation efficiency of CE.

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