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Development and validation of a quantitative assay for raloxifene by capillary electrophoresis

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Abstract

The migration behavior of raloxifene was investigated by capillary electrophoresis (CE). The influence of different parameters (nature and concentration of the running buffer, pH and applied voltage) on migration time, peak symmetry and efficiency was systematically investigated. A buffer consisting of 20 mM acetate buffer of pH 4.5 was found to provide a very efficient and stable electrophoretic system for the analysis of raloxifene. The optimized method was validated with respect to precision, linearity, limits of detection and quantification, accuracy and robustness. The applicability of the assay was demonstrated by analyzing this drug in human plasma and pharmaceutical preparations.

Keywords: Raloxifene; Capillary electrophoresis; Validation; Human plasma; Pharmaceuticals

1. Introduction

Raloxifene is a polyhydroxylated non-steroidal compound with a benzothiophene core. It is an estrogen agonist in bone, where it exerts an antiresorptive effect. The results of several large clinical trials have shown that raloxifene reduces the rate of bone loss at both distal sites and in the spinal column and may increase bone mass at certain sites [1]. The drug has beneficial actions on lipoprotein metabolism, reducing both total cholesterol and LDL; however, HDL is not increased unlike with estrogen-replacement therapy. Pre-clinical studies indicate that raloxifene has an antiproliferactive effect on estrogen receptor (ER)-positive breast tumors and on the proliferation of ER-positive breast cancer cell lines and significantly reduces the risk of ER-positive but not ER-negative breast cancer [2]. Adverse effects include deep vein thrombosis, pulmonary embolism and leg cramps [3].

Raloxifene is adsorbed rapidly after oral administration and has an absolute bioavailability of about 2%. The drug has a half-life of about 28 h and is eliminated primarily in the faeces after hepatic glucuronidation [3]. Mono- and di-oxygenated metabolites of raloxifene have been recognized by microsomal incubation

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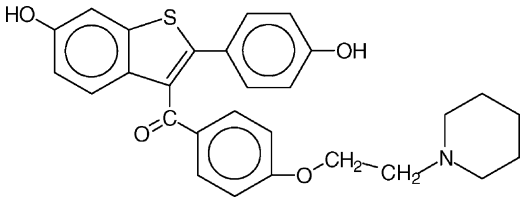
and receptor binding using fast LC/fast scanning MS [4].



There are very few methods described in the literature for the determination of raloxifene. All of them use high-performance liquid chromatography (HPLC). The drug has been determined in human plasma using the LC/MS/MS technique [5]. Reverse phase HPLC with UV detection has been proposed for quantifying raloxifene hydrochloride [6,7].

Capillary electrophoresis (CE) is gaining widespread acceptance as a standard analytical technique in pharmaceutical analysis owing to its many advantageous features, such as extremely high efficiency, high resolution, rapid analysis and small consumption of sample and reagents. CE has been employed in the determination of a variety of compounds including, pharmaceuticals, proteins, polymers and food constituents [8,9]. To our knowledge, however, no CE method has been described for the determination of raloxifene.

The purpose of this investigation was to study the electrophoretic behavior of raloxifene in order to develop a simple and rapid CE method. The assay was validated by determining its accuracy, precision, linearity, specificity and robustness. The method has been satisfactorily applied to the determination of raloxifene in pharmaceutical preparations and human plasma.

2. Experimental

2.1. Reagents

Raloxifene hydrochloride was obtained by extracting tablets of Evista (Lilly, Madrid, Spain) or Optruma (Pensa, Barcelona, Spain) with methanol. Human plasma and mesityl oxide were purchased from Sigma (St. Louis, MO, USA). The organic solvents acetonitrile, ethanol, methanol, hexane, 1,2-dichloroethane, dichloromethane and trichloromethane were of HPLC grade (Romil, Loughborough, Leicestershire, UK); other chemicals were of analytical grade. Ultrapure water from a Milli-Q plus system (Millipore Ibérica, Madrid, Spain) was used throughout. Acetate, citrate, phosphate, ADA (*N*-[2-acetamido]-2-iminodiacetic acid) and MES (2-[*N*-morpholino]ethanesulfonic acid) buffers were prepared by adjusting to the desired pH with sodium hydroxide or hydrochloric acid solutions.

2.2. Apparatus

Separations were made with a Spectraphoresis 1000CE (Thermo Separation Products, San Jose, CA, USA) equipped with UV detection. All experiments were carried out in fused-silica capillaries of 75 μm i.d., total length 80 cm, distance between injection and detection 50 cm. The wavelength of the UV detector was set at 286 nm. Sample injection was carried out in a hydrodynamic mode during 3 s with a vacuum of 40.6 kPa. The CE system was interfaced with a microcomputer. Chrom Card software (Thermo) was employed for integration and data handling.

2.3. Electrophoretic procedure

When a new capillary was used, the capillary was conditioned by first rinsing with 1 M sodium hydroxide for 10 min, then with 0.1 M sodium hydroxide for 10 min, followed by pure water for 10 min and finally with the running electrolyte for 10 min. To ensure reproducibility, the capillary was washed every day with 0.1 M sodium hydroxide, followed by the running buffer for 10 min, and then equilibrating with the buffer for 2 min, while applying the separation voltage. Between each injection, the capillary was rinsed with fresh electrolyte solution (2 min). Rinsing for 2 min was sufficient to guarantee that the contents of the previous injection were eliminated and that the capillary was filled with fresh electrolyte.

Peak identity confirmation and peak purity determination were performed with a P/ACE model 5500 instrument (Beckman Instruments, Palo Alto, CA, USA) equipped with a diode array detector.

Electroosmotic flow was determined from the migration time of mesityl oxide, which was considered to be neutral throughout the entire sequence of buffers used. Electrophoretic mobilities were calculated as the

difference between the apparent mobility of the analyte and the mobility of the neutral marker.

2.4. Determination of raloxifene in human plasma

The plasma samples were spiked with appropriate amounts of raloxifene to obtain a final concentration within its therapeutic range. A volume of 300 μl of each sample was placed in a test tube and 300 μl of methanol were added. The mixture was vortexed for 1 min and allowed to stand for 5 min and then centrifuged for 2 min. The supernatant was extracted with 5 ml of 1,2-dichloroethane. After mixing on a Vortex mixer for 5 min and subsequent centrifugation at 2500 × *g* for 2 min, the 2-dichloroethane phase was transferred with a pipette into a rotary evaporator and evaporated to dryness. The residue was dissolved in 2 ml of methanol. This solution was used to fill the sample vial.

The plasma samples were also analyzed using an HPLC Beckman Coulter (Fullerton, CA, USA) instrument. Chromatography was performed on a 5 μm Ultrasphere C_{18} column (250 mm × 4.6 mm i.d.) (Beckman). The mobile phase was acetonitrile/10 mM SDS (11:9, pH 7) and detection at 286 nm.

2.5. Determination of raloxifene in pharmaceutical preparations

The pills were finely powdered and weighed. An amount of this powder, equivalent to about 60 mg of raloxifene was accurately weighed and shaken with 100 ml of methanol. The solution was filtered and the residue was washed with methanol (two portions of 25 ml). The combined filtrate and washings was diluted with ultrapure water to 250 ml in a calibrated flask to obtain a solution of 240 μg ml^{-1}.

3. Results and discussion

3.1. Method development

3.1.1. Effect of buffer pH

Manipulation of buffer pH is a key strategy for optimizing the separation of ionizable analytes in CE because buffer pH determines the extent of the ionization of each analyte and the magnitude of the electroosmotic flow that is directed towards the negative end of fused-silica capillary.

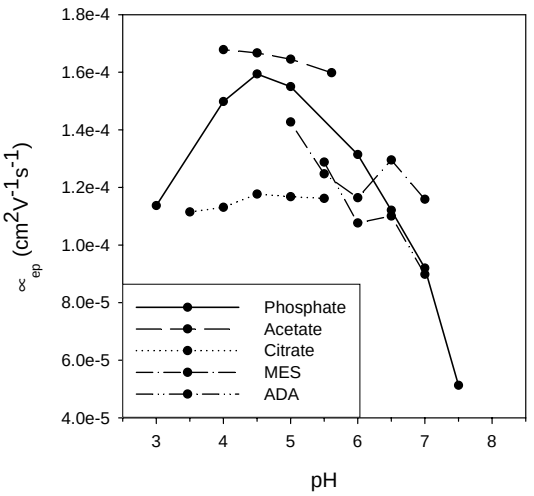


Fig. 1. Effect of pH on the electrophoretic mobility of raloxifene. Electrophoretic electrolyte: phosphate, acetate, citrate, ADA and MES. Effective capillary length, 50 cm; total length 80 cm. Applied voltage, 20 kV. Hydrodynamic injection, 1 s at 40.6 kPa.

The electrophoretic mobility of raloxifene as a function of electrolyte pH in the range 3.0–7.5 is illustrated in Fig. 1. A buffer pH higher than 7.5 could not be used because the peak of raloxifene was very poorly developed. As expected, raloxifene is positively charged at pH ≤ 4.5 and its electrophoretic mobility continuously decreased with increasing buffer pH.

3.1.2. Effect of buffer nature

Five different aqueous buffers were tested: acetate, citrate, phosphate, ADA and MES, all adjusted to the pH range 3.0–7.5 were compared. Acetate buffer led to the highest efficiency of the raloxifene peak. Since acetate buffer of pH 4.5 provided the best results, this buffer was selected for the further studies (Fig. 2).

3.1.3. Effect of ionic strength

The ionic strength of the run buffer was tested at a constant pH of 4.5. As the concentration of acetate buffer was changed from 5 to 80 mM, an increase in the migration time was obtained. We suggest that such an effect may be related to the lower electroosmotic flow resulting from a decrease in the zeta potential at the capillary wall-solution interface. The efficiency of

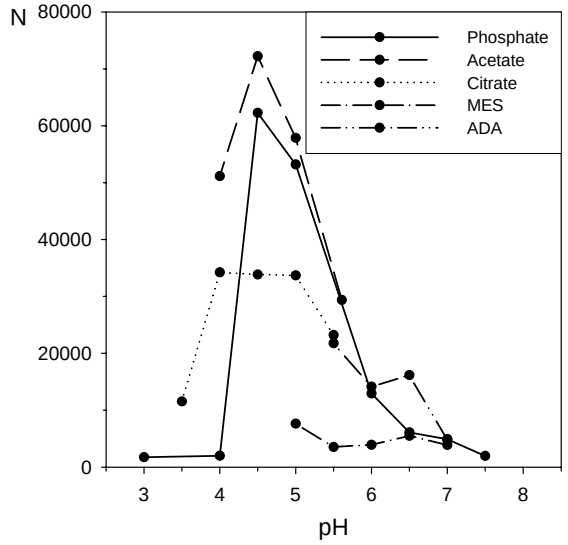


Fig. 2. Influence of the composition of the electrophoretic electrolyte on the efficiency of raloxifene peak: phosphate, acetate, citrate, ADA and MES. Buffer concentration, 20 mM. Other conditions as for Fig. 1.

the raloxifene peak improved with increasing buffer concentration up to 20 mM, and then decreased when higher concentrations were used.

3.1.4. Effect of applied voltage

The applied voltage was varied in the range 5–20 kV. Using 20 mM acetate buffer at pH 4.5 as running electrolyte, the increase of applied voltage led both to shorter migration times and shaper peaks. However, higher voltages also exhibited higher currents and increased Joule heating. To limit this heating inside the capillary the maximum applied voltage was chosen from an Ohm plot. The voltage was 12 kV (current ca. 11 μA).

3.1.5. Injection time

The length of the sample injection time was examined in an attempt to attain high sensitivity. Sample solutions were hydrodynamically injected at a vacuum of 40.6 kPa while the injection time was varied from 0.5 and 5.0 s. Satisfactory linearity between peak area and injection time (analyte quantity) was achieved within the range 0.5–3.0 s. The injection time selected was 3 s, which correspond to a plug length of ca. 3% of the whole capillary length.

3.2. Method validation

Appropriate method validation information concerning new analytical techniques for analyzing pharmaceuticals is required by regulatory authorities. Validation of such methods includes assessment of the stability of the solutions, linearity, precision, detection and quantification limits, accuracy and robustness.

3.2.1. Stability of the solutions

Although this test is often considered as a part of ruggedness of the procedure, it should be carried out at the beginning of the validation procedure because it conditions the validity of the data of the others tests.

The response factor of raloxifene standard solutions was found to be unchanged for up to 15 days. Less than 0.3% concentration difference was found between the solutions freshly prepared and those aged 15 days. The solutions can therefore be used within this period without the results being affected.

3.2.2. Linearity

External calibration was used because no improvement was observed when an internal standard was used. The linearity between peak area and concentration was tested by constructing a calibration curve from three sets of standard solutions of raloxifene at eleven different concentrations, ranging from 0.12 to 36 μg ml^{-1}. Linear regression of the calibration curve gave a linear equation, $Y = (141067 \pm 1294)X - (70332 \pm 23700)$, with a correlation coefficient (r^2) of 0.9994.

3.2.3. Accuracy and precision

The accuracy of the method was tested with several synthetic samples of raloxifene with different concentrations. The results obtained are shown in Table 1,

Table 1
Recovery and precision data in the determination of raloxifene in synthetic samples

Concentration added (μg ml^{-1})	Concentration found[a] (μg ml^{-1})	Migration time[a] (min)
0.6	0.57 ± 0.08	9.62 ± 0.06
1.2	1.19 ± 0.02	9.64 ± 0.05
2.4	2.43 ± 0.06	9.92 ±0.06
12.0	11.91 ± 0.11	9.55 ± 0.08

[a] Mean of 11 concentrations ± S.D.

from which is clear that both the recoveries and repeatabilities are excellent.

The inter-day precision of the method was studied by analyzing two sets of three identical samples containing 1.2 and 2.4 μg ml^{-1}. On five consecutive days, each sample was injected six times every day. The R.S.D. for the peak area were 3.72 and 4.84% at the concentration level of 1.2 and 2.4 μg ml^{-1}, respectively. The inter-day precision of the migration time, expressed as R.S.D., was 2.35%.

3.2.4. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD was evaluated from four independent samples, which were spiked with raloxifene in order to produce a peak height close to three times the base line noise. The LOD was estimated by taking three times the standard deviation of the peak areas obtained from these solutions and calculating the corresponding concentration. The LOD obtained was 6.1 ng ml^{-1}.

The LOQ is defined as the level at, or above, which the measurement precision is satisfactory for quantitative analysis. In our case, LOQ was estimated by taking 10 times the standard deviation of the peak areas obtained from the four samples and subsequently calculating the corresponding concentration. The LOQ was 20.5 ng ml^{-1}.

3.2.5. Robustness

Robustness is defined as the capability of an analytical procedure to remain unaffected by small but deliberate changes in the method parameters. A good efficiency of the raloxifene peak was always obtained with variations of ±10% of the optimum value of the electrophoretic parameters, buffer pH, buffer concentration and applied voltage.

3.3. Applications

The CE assay is characterized by long-term stability and reproducibility. More than 2000 analyses could be performed without having to replace the capillary. To demonstrate the usefulness at the procedure for the determination of raloxifene, pharmaceutical formulations and human plasma were analyzed.

The linearity and precision of the assay in plasma were tested using spiked samples. The linearity was confirmed in the range 0.2–30 μg ml^{-1}. The correlation coefficient of the regression lines was 0.999 or higher. The precision of the method was assessed by determining three concentrations within the range 3–10 μg ml^{-1} in three independent series of samples. Table 2 summarizes the results obtained using the CE method and a HPLC procedure [6]. Inter-day precision data were observed over a period of five working days; R.S.D. < 6.41% were always obtained. Fig. 3 shows an electropherogram of a spiked plasma sample, where it can be observed that there are no endogenous interfering peaks in the migration window of raloxifene.

The proposed method was also used to determine raloxifene in pharmaceutical preparations. Additives and excipients did not interfere. The quantification of raloxifene in pharmaceuticals was carried out from the calibration graph obtained using the standard solutions. The data in Table 3 show that the assay results were in good agreement with the labeled content and the HPLC method. The recoveries studies were carried out with the two pharmaceuticals fortified with raloxifene at 30, 100 and 150% of the label claim. Each sample was analyzed seven times; the recovery percentages obtained were in the range 96.1–98.7% with R.S.D. between 1.6 and 1.8%.

Table 2
Analytical recovery and precision of CE method for serum samples

Amount added (μg ml^{-1})	Amount found[a] (μg ml^{-1}) CE method	Amount found[a] (μg ml^{-1}) HPLC method
3.43	3.37 ± 0.14	3.36 ± 0.47
6.00	5.54 ± 0.29	5.64 ± 0.45
9.60	9.28 ± 0.12	9.25 ± 0.35

[a] Mean of four determinations ± S.D.

Table 3
Determination of raloxifene in pharmaceutical formulations by capillary electrophoresis

Sample (supplier)	Nominal content (mg per pill)	Raloxifene found[a] (mg per pill)
Evista (Lilly)	60	59.3 ± 2.7
Optruma (Pensa)	60	58.1 ± 2.4

[a] Mean of seven determinations ± S.D.

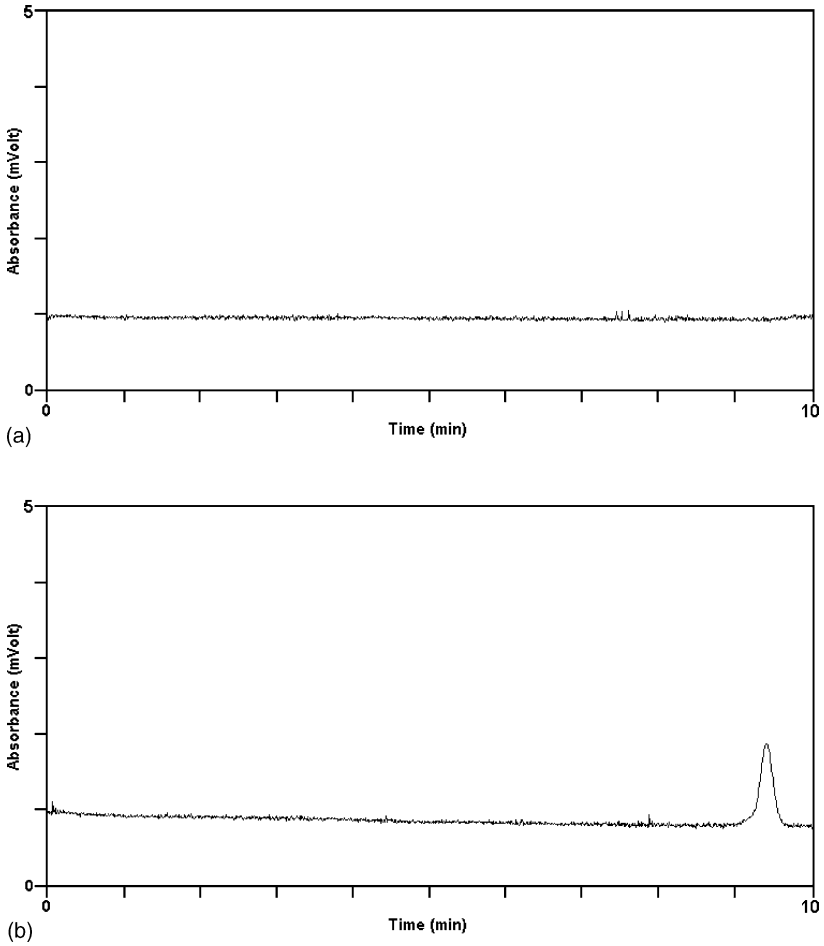


Fig. 3. Electropherogram obtained from (a) human serum blank; (b) serum with raloxifene (0.94 μg ml^{-1}). The electrolyte was 20 mM acetate buffer, pH 4.5. Applied voltage, 12 kV. Hydrodynamic injection was performed for 3 s at 40.6 kPa. Other conditions as for Fig. 1.

4. Conclusions

A simple, rapid, efficient and reliable method for the CE determination of raloxifene has been developed using 20 mM acetate buffer adjusted to pH 4.5 as electrophoretic electrolyte. The lowest concentration of the calibration graph for raloxifene was 2.5×10^{-7} M. The CE assay was supported by method validation, which demonstrated excellent precision, accuracy, linearity and robustness. The usefulness of this method is demonstrated by the excellent results obtained in the determination of raloxifene in human plasma and pharmaceutical preparations.

The present method compared with the reported HPLC methods, offers several advantages such as wider concentration range and lower reagent consumption. In addition, its sensitivity is higher than that of the two HPLC which use photometric detection [6,7], although less than with a high-throughput LC/MS/MS technique [5].

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References

[1] P.D. Delmas, N.H. Bjarnason, B.H. Mitlak, A.C. Ravoux, A.S. Shah, W.J. Huster, M. Draper, C.N. Christiansen, Engl. J. Med. 337 (1997) 1641–1647.
[2] T. Hol, M.B. Cox, H.U. Bryant, M.W. Draper, J. Womens Health 6 (1997) 523–531.
[3] J.G. Hardman, L.E. Limbird (Eds.), Goodman and Gilman's, The Pharmacological Basis of Therapeutics, 10th ed., McGraw-Hill, New York, 2001.
[4] H.K. Lim, S. Stellingweif, S. Sisenwine, K.W. Chan, J. Chromatogr. A 831 (1999) 227–241.
[5] J. Zweigenbaum, J. Henion, Anal. Chem. 72 (2000) 2446–2454.
[6] P. Nandini, W.P. Jayant, Indian Drugs 38 (2001) 591–592.
[7] Q. Wang, H.M. Zhang, Z.G. Yu, Shenyang Yaoke Daxue Xuebao 19 (2002) 105–108.
[8] S.M. Lunte, D.M. Radzik, Pharmaceutical and Biomedical Applications of Capillary Electrophoresis, Elsevier, Oxford, 1996.
[9] H. Shintani, J. Polonsky, Handbook of Capillary Electrophoresis Applications, Chapman & Hall, London, 1997.