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HPLC-fluorescence determination of unconjugated estrogens in pharmaceuticals[1]

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Abstract

A fluorimetric liquid chromatographic method ($\lambda_{ex} = 280$ nm; $\lambda_{em} = 312$ nm) was developed for measurements of unconjugated estrogens (estradiol and estriol) in pharmaceutical dosage forms using a reversed-phase column with water–acetonitrile at different composition as mobile phase. The in vitro release profiles of three different estradiol transdermal therapeutic systems were determined through a medical-grade silicone rubber subdermal implant material membrane, using a modified Franz diffusion apparatus at 37°C in presence of PEG 400. The HPLC method possesses advantages of rapidity, simplicity and accuracy.

Keywords: Estradiol and estriol determination; Reversed-phase liquid chromatography; Transdermal delivery system; Fluorescence detection

1. Introduction

Estrogens are naturally occuring hormones formed from androgen precursors in the ovarian follicles of premenopausal women, under the influence of the pituitary. These hormones are given for replacement therapy in deficiency states, for menopausal and postmenopausal disorders and for contraception. They are administered orally, subcutaneously via an implant, locally as vaginal cream or tablets, intramuscularly, or transdermally via a skin patch. They are also used in the management of breast cancer in menopausal and postmenopausal women and in the management of prostate cancer [1].

For the analysis of estradiol and estriol in pharmaceuticals, methods based on electroanalytical procedures [2], liquid chromatography (HPLC) with UV detection [3,4], thin-layer chromatography [3], alkaline phosphatase and chemiluminescent modified procedures [5] have been proposed.

To provide continuous drug infusion through an intact skin, several transdermal therapeutic systems have been developed for topical application onto the intact skin surface to control the delivery of the drug and its subsequent permeation through the skin tissue [6,7]. The develop-

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ment of transdermal drug delivery (TDD) systems of the female and male hormone without the side effects of frequent oral administration is very important. It is therefore essential to have a simple and reliable in vitro test method that can measure the consistency and uniformity of drug release. Recently, many different in vitro release (dissolution) testing procedures have been developed for transdermal products (patches), using the high performance liquid chromatography (HPLC) analyses with UV detection [8,9] and by spectrophotometry [10].

In the present paper, HPLC with fluorescence detection ($\lambda_{ex} = 280$ nm; $\lambda_{em} = 312$ nm) is proposed for a rapid and reliable quality control of estrogens (estradiol and estriol) in pharmaceutical formulations. Moreover the purpose of this study was to evaluate if the described method would be suitable to determine the amounts of estradiol released from transdermal therapeutic systems in in vitro experiments.

2. Experimental

2.1. Materials

Estradiol and estriol were obtained from Sigma (St. Louis, MO). Acetonitrile and methanol for chromatography were HPLC grade, from Romil (Delchimica Scientific Glassware, Napoli, Italy). Double distilled water was used. Polyethylene glycol (PEG) 400 were from Fluka (Buchs, Switzerland). Other chemicals were from Carlo Erba Reagent (Italy). The Silastic®$_{BRAND}$ sheeting non-reinforced (8×6 inches$^2 \times 0.10$, a medical-grade silicone rubber subdermal implant material) was from Dow Corning Corporation (Midland, MI). The patches Estraderm TTS 50 (lot no. 340000) marketed by Ciba-Geigy S.p.A. (Origgio, Varese, Italy), Estroclim 50 (lot no. 338500) marketed by Sigma-tau, Industrie Farmaceutiche Riunite S.p.A, (Roma, Italy), and Epiestriol 50 (lot no. B00E57) marketed by Rotta Research Laboratorium S.p.A. (Monza, Milano, Italy), are round in shape with an area of 10 cm^2, containing 4 mg of estradiol per patch and providing in vivo delivery of 0.05 mg day^{-1}.

2.2. Apparatus

The liquid chromatograph comprised a Varian 2010 pump, a Jasco FP-920 fluorescence spectrophotometer, operating at an emission wavelength of 312 and 410 nm with an excitation wavelength of 280 nm, connected to a computer AcerView 34TL. The integration program Borwin was used. Manual injections were carried out using a Rheodyne model 7125 injector with 50 μl sample loop. The solvents were degassed on line with a degasser model ERC-3312 from Erma (Tokyo, Japan). A Beam Boost model C6808 photoreactor (ICT, Frankfurt, Germany) was arranged on-line between the analytical column and the detector. The eluate was irradiated on-line in capillary PTFE tubing (20 m × 0.3 mm i.d.) in a crocheted geometry with an 8-W low-pressure mercury lamp with the main spectral emission at 254 nm. Sonarex Super RK 102 (35 KMz) Bandelin (Berlin, Germany) equipment with termostatically controlled heating (30–80°C) was used for ultrasonication.

2.3. Solutions

Stock solutions of estrogens were prepared in mobile phase (concentration under calibration graphs). Saline solution was prepared dissolving 9.0 g of sodium chloride in water to make 1000 ml. Saline pH 7.4 phosphate buffered solution was prepared dissolving 1.38 g of disodium hydrogen orthophosphate, 0.19 g of potassium dihydrogen orthophosphate and 8.0 g of sodium chloride in sufficient water to produce 1000 ml. The 50% (v/v) PEG 400 was prepared in saline pH 7.4 phosphate buffered solution.

2.4. Chromatographic conditions

The HPLC separations were performed at 35°C using a stainless steel column Tracer Spherisorb 5 ODS_2 (250 × 4.6 mm i.d.) and Phenomenex Prodigy 5 ODS_2 (250 × 4.6 mm i.d.) under isocratic conditions. For routine analyses a mobile phase consisting of water–acetonitrile (58:42, v/v) for estradiol and (64:36, v/v) for estriol was used at a flow rate of 1.0 ml min^{-1}.

2.5. Calibration graphs

Standard solutions of estradiol (42.50–1700 ng ml^{-1}) and estriol (6.42–642 ng ml^{-1}) were prepared in mobile phase. Triplicate injections for each standard solution were made and the peak-area was plotted against the corresponding estrogen concentration to obtain the calibration graphs.

2.6. Analysis of pharmaceutical formulations

2.6.1. Sample preparation

The sample handling was carried out according to the nature and composition of the commercial dosage forms.

2.6.1.1. Estradiol (transdermal delivery systems). At first the membrane TDD systems were perforated with a needle, then every patch (membrane permeation-controlled drug resevoir type TDD systems and matrix diffusion-controlled drug dispersion type TDD systems), equivalent to 4 mg of drug, was treated with 50 ml of a mixture of acetonitrile–methanol (70:30, v/v) under ultrasonication for 20 min at room temperature. Then a 0.1-ml aliquot of the obtained solution was diluted to 10 ml with the mobile phase. At last the resulting solution was filtered with 0.45 μm nylon 25 mm filter and used for the HPLC analysis in comparison with an appropriate standard solution (0.80 μg ml^{-1}).

2.6.1.2. Estriol (tablets). An amount of powdered tablet equivalent to about 1.0 mg of estriol was extracted with methanol (3×30 ml) and diluted to 100 ml. After filtration through paper the clarified solution was diluted to obtain a sample solution (about 0.40 μg ml^{-1}) in the mobile phase. The resulting solution was filtered with 0.45 μm nylon 25 mm filter and then subjected to the HPLC analysis in comparison with an appropriate standard solution (0.48 μg ml^{-1}).

2.6.1.3. Estriol (cream). An amount of the cream equivalent to about 0.125 mg estriol was dissolved in 250 ml of a mixture methanol–acetonitrile (90:10, v/v) by ultrasonication at room temperature for 10 min and a 1-ml aliquot of the resulting clear solution was diluted to 10 ml with mobile phase. The resulting suspension was filtered with 0.45 μm nylon 25 mm filter and the obtained solution was used for the HPLC analysis in comparison with an appropriate standard solution (0.05 μg ml^{-1}).

2.7. In vitro release of estradiol

2.7.1. Sample preparation

The transdermal patch was held in position using a diffusion cell, such as the Franz diffusion cell [6], with the cap cell of about 6 cm diameter size. The pach sandwich was stuck to a medical-grade silicone rubber subdermal implant material membrane instead of the skin. The release profile was determined at 37°C. The solution receptor compartment (about 93 ml) were removed at 3, 6, 24, 48, 72 and 96 h intervals replacing the medium. The removed solution was diluted to 100 ml with the medium and 1.0-ml aliquot of the obtained solution was diluted to 10 ml with the mobile phase. The resulting solution was filtered with 0.45 μm nylon 25 mm filter and then injected in the chromatograph. The amount of estradiol was determined by direct comparison with an estradiol standard solution (0.05 μg ml^{-1}).

3. Results and discussion

3.1. Chromatography

Chromatographic separations were carried out under isocratic conditions at 35°C on a reversed phase column 5 ODS_2. The effects of composition and pH of the mobile phase on the resolution and fluorescence intensity of estrogens were investigated. A mixture water–acetonitrile (58:42, v/v) and (64:36, v/v) for estradiol and estriol, respectively, at a flow rate of 1.0 ml min^{-1}, was found to be suitable for fluorescence detection ($\lambda_{ex} = 280$ nm; $\lambda_{em} = 312$ nm). Representative HPLC separation of a mixture of estradiol and estriol is reported in Fig. 1a.

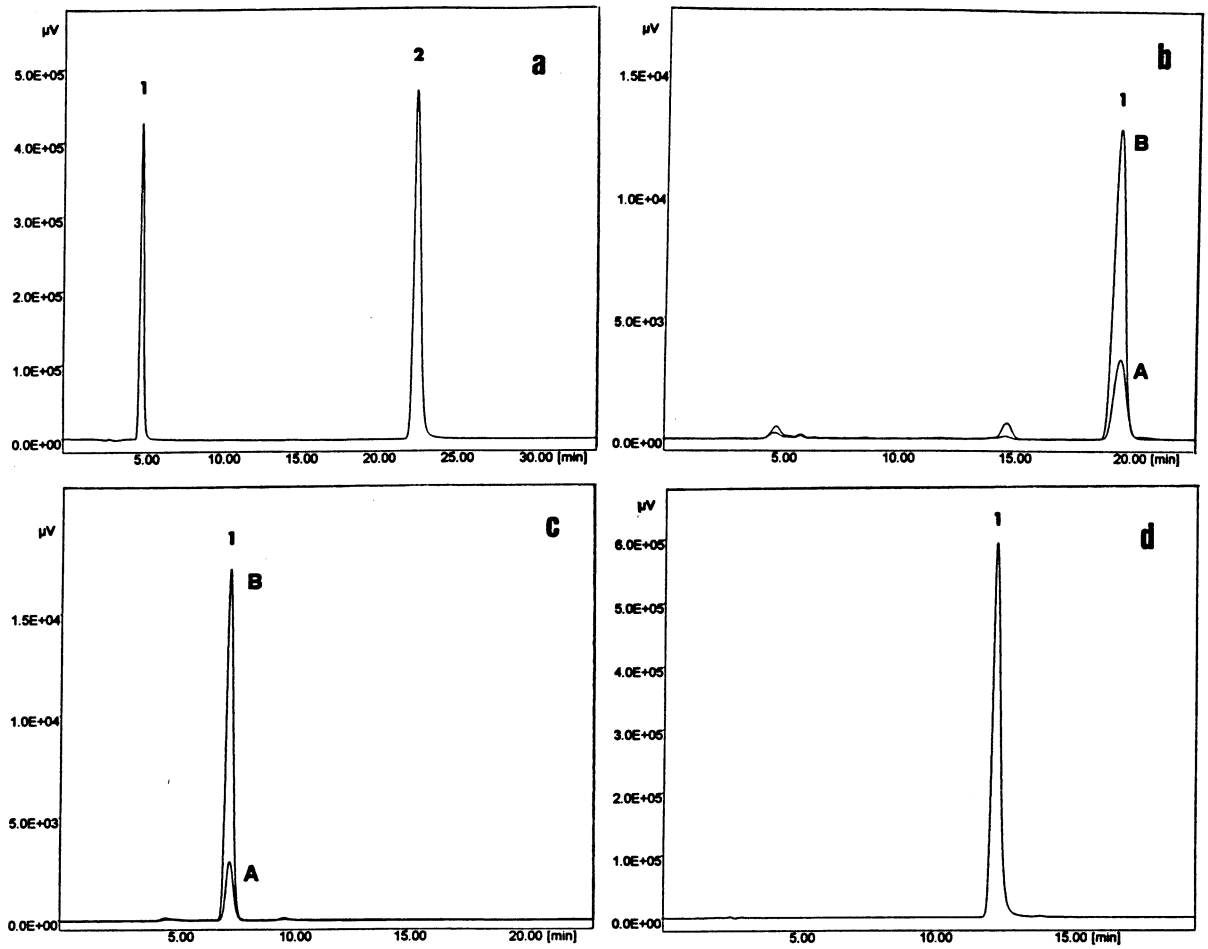

Fig. 1. Representative HPLC separation at 35°C of estrogens. (a) Peaks: 1. estriol; 2. estradiol. Column Tracer Spherisorb 5 ODS_2 (250 × 4.6 mm i.d.). Mobile phase: water–acetonitrile (64:36, v/v) at a flow rate of 1.0 ml min^{-1}. Fluorescence detection: $\lambda_{ex} = 280$ nm; $\lambda_{em} = 312$ nm; (b) Peak: 1. estradiol (30 µg ml^{-1}) with on-line photoreactor swiched (A) off and (B) on. Column Phenomenex Prodigy 5 ODS_2 (250 x 4.6 mm i.d.). Mobile phase as in a. Fluorescence detection: $\lambda_{ex} = 280$ nm; $\lambda_{em} = 410$ nm; (C) Peak: 1. estriol (15 µg ml^{-1}) with on-line photoreactor switched (A) off and (B) on. Column and detection as in b. Mobile phase as in a; (D) Peak: 1. estradiol (TDD systems). Column as in a. Mobile phase: water–acetonitrile (58:42, v/v). Detection as in a.

The use of HPLC with fluorescence detection in combination with a postcolumn on line photochemical derivatization was investigated to achieve higher detector response and to enhance the selectivity in the HPLC analysis. The photochemical reactor allows irradiation of the eluent coming from the analytical column by UV-light ($\lambda = 254$ nm) before it enters in the detector. The irradiation of the analyte can cause a modification of its structure which can lead to species offering other detection behaviour than the parent compound. As can be seen in Fig. 1b,c, estradiol and estriol fluorescence intensity increases significantly at $\lambda_{em} = 410$ nm with $\lambda_{ex} = 280$ nm. The effects do not appear at $\lambda_{em} = 312$ nm with $\lambda_{ex} = 280$ nm, but in these conditions the fluorescence intensity is considerably higher. The detection limit (signal/noise ratio = 3) at $\lambda_{em} = 312$ nm was about 40 fmol and 4 amol for estradiol and estriol, respectively.

3.2. Analysis of pharmaceutical formulations

Under the described chromatographic conditions (Fig. 1a,d) a linear relationship between the peak-area (y; μV) and the drug concentration (C: 42.50–1700 and 6.42–642 ng ml^{-1} for estradiol and estriol, respectively) was obtained ($y = 16481C - 231862$; $r = 0.9998$; $n = 6$ for estradiol and $y = 12335C - 33855$; $r = 0.9999$; $n = 6$ for estriol). The satisfactory within-run precision of the method was indicated by the relative standard deviations of the peak-area from the replicate ($n = 8$) analyses of a single standard solution of estradiol (0.80 μg ml^{-1}; RDS 0.07%) and estriol (0.16 μg ml^{-1}; RDS 0.38%).

The method was applied to the HPLC analyses of commercial formulations of estradiol (TDD systems) and estriol (tablets and cream). The results obtained (Table 1) were found to be in agreement with the claimed content of the drugs. The other ingredients of the formulations did not interfere with the analysis. The accuracy of the methods was verified by analysing commercial samples spiked with known amounts of drugs (20% of the claimed content); essentially quantitative recoveries were obtained for both estradiol ($100.62 \pm 0.75\%$)) and estriol ($99.50 \pm 1.0\%$). An example of chromatogram obtained from commercial dosage form is illustrated in Fig. 1d.

Table 1
Results by HPLC analysis of estradiol and estriol in pharmaceutical formulations

Drug	Formulation[a]	Found[d]	RSD%
Estradiol	TDD systems[b]	99.98	0.93
	TDD systems[b]	99.25	1.06
	TDD systems[c]	101.50	0.70
Estriol	Tablets	99.37	0.75
	Cream	97.28	1.20

[a] Other ingredients. [b] TDD systems: ethanol, hydroxypropylcellulose; [c] TDD systems: acrylic copolimers; Tablets: amylopectin, magnesium stearate, starch, lactose; Cream: palmitate-stearate polyoxyethyleneglicol, polyoxyethylenate oleic glycerides, neutral sature triglycerides, propylene glycol, benzoic acid, purified water.
[d] Mean of five determinations and expressed as a percentage of the claimed content.

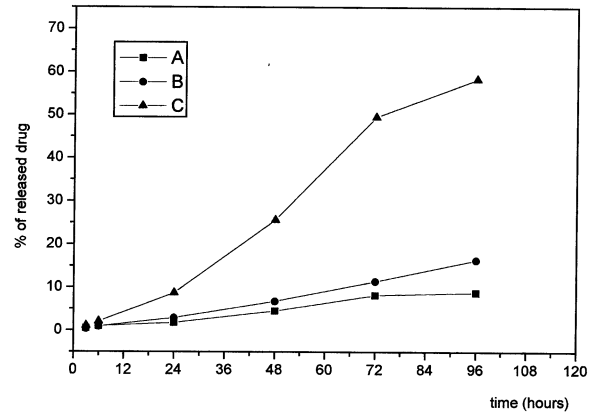

Fig. 2. Effect of solvent on the permeation of estradiol from 4 mg TDD systems (Estroclim 50) through a medical-grade silicone rubber subdermal implant material membrane, mounted on a diffusion apparatus (modified Franz cell): (A) methanol; (B) saline sodium chloride solution (0.9% w/v); (C) saline pH 7.4 phosphate buffered solution-PEG 400 (50:50, v/v).

3.3. In vitro release of estradiol

The percentage of daily released drug in comparison with the nominal value (0.05 mg day^{-1}) from three different TDD systems was evaluated and compared at 37°C through a medical-grade silicone rubber subdermal implant material membrane, using a modified Franz cell as a diffusion apparatus. To achieve optimum conditions the effect of the solvent placed into the receptor compartment was investigated (Fig. 2). As can be seen from the dissolution profile, the release of the drug from same patche type (Estroclim 50), choosen as an example, increased using the 50% (v/v) PEG 400 solubilizer added to saline pH 7.4 phosphate buffered solution. The results of the dissolution profiles of three different series of transdermal therapeutic systems, using 50% (v/v) PEG 400, under identical conditions, are reported in Fig. 3. The total percentage of estradiol released is quite low, even after 96 h for both membrane systems and constant and linear for 3 and 4 days for the membrane patches and for the matrix patches, respectively. This is in agreement with the drug administration. For estrogen replacement in menopausal and postmenopausal woman estradiol patches are generally changed every 3–4 days.

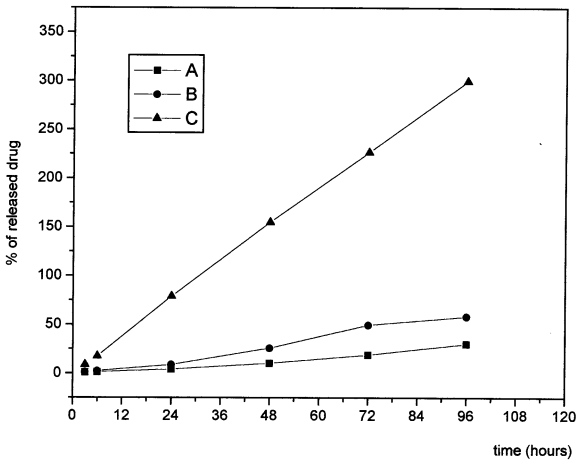

Fig. 3. In vitro dissolution profile of TDD systems, containing 4 mg of estradiol, through a medical-grade silicone rubber subdermal implant material membrane, mounted on a diffusion apparatus (modified Franz cell) at 37°C: (A) membrane permeation-controlled TDD systems (Estraderm 50); (B) membrane permeation-controlled TDD systems (Estroclim 50); (C) matrix dispersion-type TDD systems (Epiestrol 50).

4. Conclusions

The proposed HPLC method with fluorescence detection was successfully applied to the sensitive and selective determination of estrogens in pharmaceutical formulations. The method possesses advantages of rapidity, accuracy and semplicity; it also provides a suitable procedure for the quality control of estradiol TDD systems. Moreover a useful in vitro release method for testing estradiol transdermal patches has been developed. This procedure was found to be simple, reliable and reproducible. The on-line photochemical derivatization at $\lambda_{em} = 410$ nm with $\lambda_{ex} = 280$ nm can be of benefit for the characterization of these hormones; applications to the analysis of estradiol in biological samples are in progress.

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