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Fluorodensitometric determination of conjugated estrogens in raw material and in pharmaceutical preparations*

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Abstract: A simple, rapid and reproducible fluorodensitometric method for the determination of conjugated estrogens has been developed. The proposed procedure includes the following steps: extraction, hydrolysis of sodium sulphate esters of estrone, equilin, equilenin and their 17-α-hydroxy derivatives, separation of the liberated 3-phenolic steroids and *in situ* measurement of fluorescence. The fluorescence emission was measured after spraying the spots of estrone and estradiol with 2,4-dinitrophenylhydrazine in sulphuric acid–ethanol medium and equilin and 17-α-dihydroequilin with phosphoric acid and sodium hydroxide solution, respectively. Equilenin and 17-α-dihydroequilenin were determined by measuring the native fluorescence. The method applied to the determination of raw material and tablets provided results which agreed well with the stated content and the requirements of USP XXI for conjugated estrogens.

Keywords: *Conjugated estrogens; fluorodensitometric determination; raw material and pharmaceutical preparation.*

Introduction

The fluorescence properties of estrogens have been widely utilized for the qualitative and quantitative analysis of estrogens in dosage forms and biological fluids [1, 2]. Fluorimetric methods for determining estrogens based on their native fluorescence [3–6] or the formation of fluorescent derivatives, mostly dansyl, have been reported. The determination of estrone, estradiol or estriol as dansyl derivatives has been carried out spectrofluorimetrically [7], fluorodensitometrically [8, 9] or by using HPLC with fluorescence detection [10]. Formation of dansyl derivatives in the analysis of conjugated or esterified estrogens has also been investigated. HPLC with fluorimetric detection of the derivatives is preceded by hydrolysis of the sulphate esters of estrone, equilin, equilenin and their 17-α-hydroxy derivatives and by reduction of the 17-keto steroids to the corresponding 17-α-hydroxyl derivatives using sodium borohydride [11]. Many fluorescence procedures have been described for the assay of estrogens based on solvent extraction or column chromatographic separation [12] followed by treatment with

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sulphuric or phosphoric acid solution or with a sulphuric acid-containing reagent, such as Kober–Ittrich [13, 14] or Liebermann–Burchard [15] reagents. The aim of this work was to develop a simple, rapid and reproducible procedure based on the *in situ* measurement of fluorescence of conjugated estrogens. The method appears suitable for the quantitative assay of conjugated estrogens in raw materials and in pharmaceutical dosage forms.

Experimental

Materials and methods

Estrone, equilin, equilenin and 17-α-estradiol were purchased from Sigma Chem. Co. (St. Louis, USA), while 17-α-dihydroequilin and 17-α-dihydroequilenin were obtained from Diosynth (Oss, Holland). Raw material of conjugated estrogens (Diosynth) contained 604 mg of a mixture of estrone, equilin, equilenin and their 17-α-hydroxy derivatives per gram. Hormoplex®, sugar-coated tablets ("Galenika", Zemun, Yugoslavia) contained 1.25 mg total conjugated estrogens. All chemicals and solvents used were of analytical grade. Chromatoplates (20 × 20 cm, precoated with 0.2 mm silica gel 60 F254) were purchased from Merck (Darmstadt, FRG). Hamilton microliter syringes were used for spotting. Solvent systems employed for TLC separation: S_1 chloroform–cyclohexane–dioxan–triethylamine (45:40:10:6) and S_2 isopropyl ether–acetone–triethylamine (80:20:10). Spraying reagents: SR_1 0.005 M dinitrophenylhydrazine in conc. sulphuric acid–ethanol mixture (1 + 1, v/v), SR_2 85% phosphoric acid in ethanol (1 + 9, v/v) and SR_3 0.1 M sodium hydroxide in ethanol. A CAMAG-TLC-Scanner (secondary cut-off filter emission >400 nm) was used.

Preparation of standard solutions

Stock solutions of 0.6 mg ml^{-1} estrone and 0.4 mg ml^{-1} equilin, equilenin, 17-α-dihydroequilin and 17-α-dihydroequilenin were prepared in chloroform. The stock solution of 0.4 mg ml^{-1} estradiol was prepared in chloroform–ethanol mixture (4 + 1). For establishing calibration curves dilutions of the stock solution were made so that the application of each of 1.5 μl for estradiol, 3 μl for estrone, equilin and 17-α-dihydroequilinin and 15 μl for equilenin and 17-α-dihydroequilenin provided a series of spots in the ranges 0.016–0.09, 0.4–2, 0.36–1.2, 0.096–0.48, 0.18–0.8 and 0.18–0.8 μg, respectively.

Preparation of sample extract

Raw material. A quantity of raw material containing 25 mg of conjugated estrogens was placed in a beaker and dissolved in 20 ml methanol. Water (20 ml) and concentrated hydrochloric acid (4 ml) were added together with some boiling clips and heated for 5 min by boiling. After cooling to room temperature the solution was transferred to a separating funnel and extracted with 2 × 10 ml and 1 × 5 ml chloroform. The combined extracts were washed with 5 ml water and passed through 1 g of anhydrous sodium sulphate into a 25 ml volumetric flask and diluted to volume with chloroform, 1.5, 3 and 15 μl of the extracts were applied to the plate.

Tablets. Twenty tablets were weighed and powdered. A quantity of powder containing 25 mg of conjugated estrogens was extracted with 70 ml of methanol and filtered quantitatively into a beaker. Water (70 ml) and concentrated hydrochloric acid (4 ml)

were added. The hydrolysis, extraction and application procedure as described for the raw material were then carried out.

Fluorodensitometry

The separation of the estrogens was carried out in solvent system S_1 followed by fluorescence detection of estrone and estradiol after spraying with reagent SR_1 and heating the plates at 80°C for 5 min, and of equilin and 17-α-dihydroequilin after spraying with reagents SR_2 and SR_3 respectively. The separation was carried out in solvent system S_2 followed by the detection of native fluorescence for equilenin and 17-α-dihydroequilenin. R_f values for estrone, equilin, 17-α-estradiol and 17-α-dihydroequilin, equilenin and 17-α-dihydroequilenin in S_1 were 0.58, 0.51, 0.44, 0.30, 0.39 and 0.25, in S_2 were 0.66, 0.61, 0.57, 0.50, 0.46 and 0.37, respectively.

Fluorescence readings were carried out at excitation and emission wavelengths of 350 and >400 nm for estrone, equilin, 17-α-estradiol and 17-α-dihydroequilin and at 340 and >400 nm for equilenin and 17-α-dihydroequilenin. The scanning conditions employed were: slit length 6 mm, slit width 1.2 mm, scan speed 1 mm s^{-1}, recorder voltage 50 mV and chart speed 50 mm min^{-1}.

The content of each estrogen per gram of raw material or per tablet was calculated using the following equations (a) or (b), respectively.

$$A \times \frac{25}{v} \times \frac{1000}{Q_x} \times 1.38, \tag{a}$$

$$A \times \frac{25}{v} \times \frac{Q}{Q_x} \times 1.38, \tag{b}$$

where, A = quantity (in mg) for each single estrogen read from the calibration curve or calculated from the corresponding regression equation, v = volume of extract (in μl) spotted on plates, Q = average weight of one tablet, Q_x = quantity of raw material or tablet powder (in mg) used in the assay and 1.38 = factor converting free estrogens to conjugate sodium salt.

Results and Discussion

Investigations with pure estrogen standards have been performed in order to establish a quantitative method based on *in situ* fluorescence measurements of estrone, equilin, equilenin and their 17-α-hydroxy derivatives separated by TLC. The fluorescence emission (>400 nm) was measured for estrone, equilin and their 17-α-hydroxy derivatives at the wavelength of excitation (350 nm) after treatment with reagents containing sulphuric acid, phosphoric acid or sodium hydroxide. It has been established experimentally that estrone and 17-α-estradiol require to be sprayed with the sulphuric acid-containing reagent and heated at 80°C for 5 min prior to the fluorescence reading. Dumazert *et al.* have reported that the presence of 2,4-dinitrophenylhydrazine in sulphuric acid–ethanol gives satisfactory sensitivity and stability of the orange colours of estrone and estradiol derivatives. However, much milder conditions using spray reagents of 85% phosphoric acid in ethanol (1 + 9, v/v) and 0.1 M sodium hydroxyde in ethanol gave enhanced fluorescence of equilin and 17-α-dihydroequilin respectively and were used to obtain reproducible *in situ* emission measurements. A five-fold increase of

intensity of fluorescence was obtained when 17-α-dihydroequilin was sprayed with sodium hydroxide rather than phosphoric acid. The native fluorescence of equilenin and 17-α-dihydroequilenin after excitation at 340 nm [17] was utilized for their determination. The *in situ* excitation spectra and fluorodensitograms of each estrogen using the conditions discussed above are presented in Figs 1 and 2.

Excellent correlations between the quantities of each estrogen applied to the plate and the peak height of the measured fluorescence emission were achieved ($r > 0.993$). Linearity of the signal with concentration was obtained up to 2, 1.2, 0.8, 0.09, 0.48 and 0.8 μg for estrone, equilin, equilenin, 17-α-estradiol, 17-α-dihydroequilin and 17-α-dihydroequilenin, respectively. Detection limits of 0.01 μg ($n = 5$) for estradiol and for

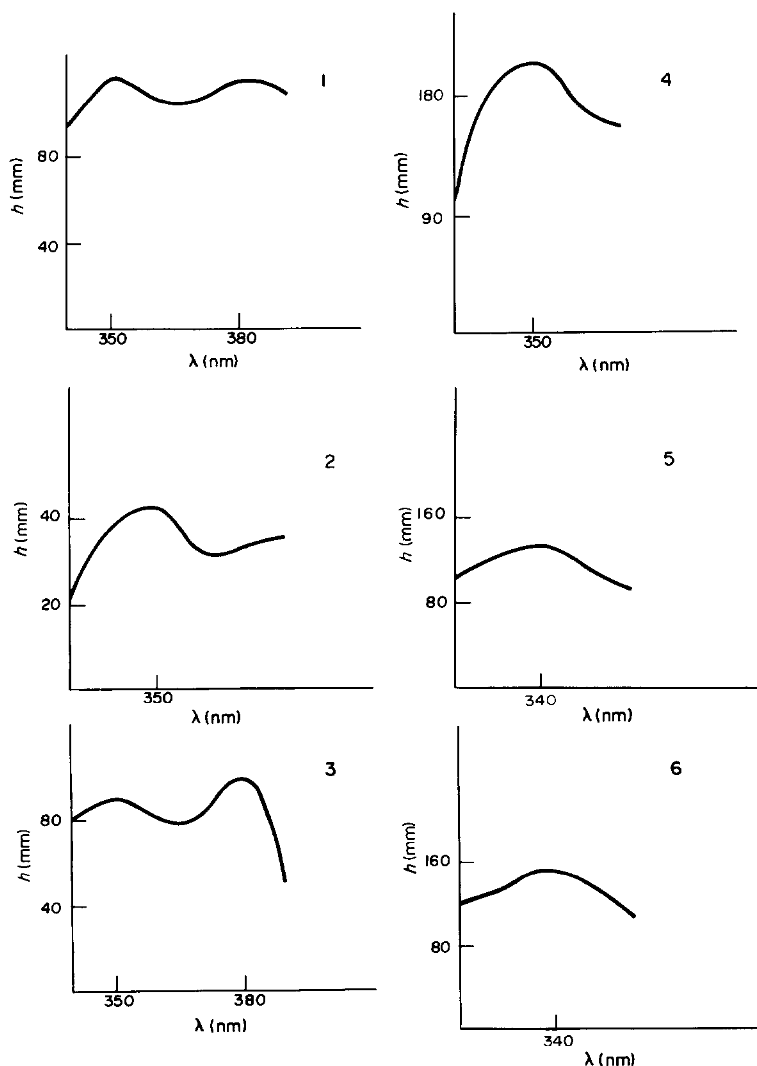

Figure 1
Excitation spectra of estrone (**1**), equilin (**2**), 17-α-estradiol (**3**), 17-α-dihydroequilin (**4**), equilenin (**5**) and 17-α-dihydroequilenin (**6**) scanned *in situ*.

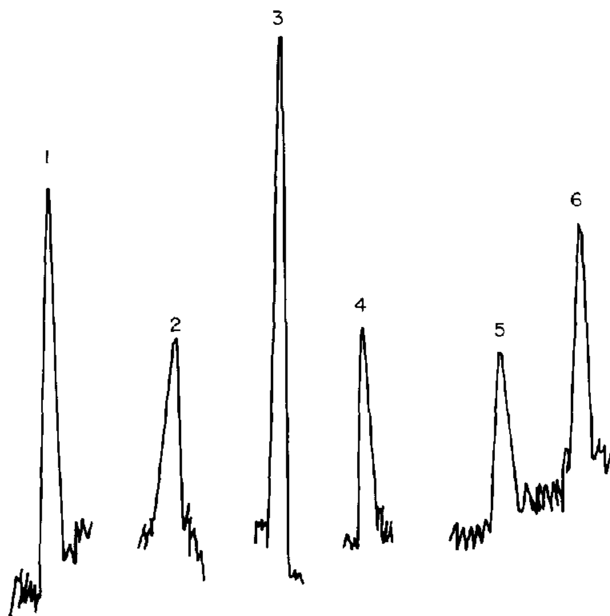

Figure 2
Fluorodensitogram of estrone (1.2 μg) (**1**), equilin (0.72 μg) (**2**), 17-α-estradiol (0.06 μg) (**3**), 17-α-dihydroequilin (0.24 μg) (**4**), equilenin (0.24 μg) (**5**) and 17-α-dihydroequilenin (0.24 μg) (**6**).

17-α-dihydroequilin and of 0.06 μg ($n = 5$) for 17-α-dihydroequilenin and the 17-keto derivatives were obtained. The statistical parameters of the calibration curves such as regression equations, coefficients of correlation (r), standard errors (S_{yx}), and Y-axis intercept errors (S_a) were $Y = 10.11 + 56.87x$, $r = 0.993$, $S_{yx} = 3.35$, $S_a = 4.19$ for estrone, $Y = 0.56 + 53.35x$, $r = 0.999$, $S_{yx} = 0.68$, $S_a = 0.67$ for equilin, $Y = -4.24 + 2048.8x$, $r = 0.998$, $S_{yx} = 2.27$, $S_a = 3.83$ for 17-α-estradiol, $Y = 5.86 + 143.26x$, $r = 0.995$, $S_{yx} = 2.73$, $S_a = 3.08$ for equilenin, $Y = -2.96 + 208.73x$, $r = 0.999$, $S_{yx} = 1.40$, $S_a = 1.19$ for 17-α-dihydroequilin and $Y = -2.38 + 211.41x$, $r = 0.999$, $S_{yx} = 0.87$, $S_a = 1.11$ for 17-α-dihydroequilenin.

Table 1
Raw material assay

Compound	Declared*	Found*	Percentage†	RSD %
Sodium estrone sulphate	334.0	364.0	57.62	7.44
Sodium equilin sulphate	180.0	181.6	28.74	6.93
Sodium 17-α-dihydroequilin sulphate	35.8	32.1	5.08	5.49
Sodium 17-α-estradiol sulphate	29.6	29.9	4.73	7.25
Sodium equilenin sulphate	16.1	15.3	2.47	4.16
Sodium 17-α-dihydroequilenin sulphate	8.6	8.8	1.40	10.59
Total amount	604.0	631.9	104.62	

*Sodium sulphate esters of estrogens expressed in milligrams per gram raw material.
†Average of seven determinations; weight found/total weight found ×100.
RSD — relative standard deviation.

The results presented in Table 1 for the content of estrogens in a sample of raw material and the total quantity of conjugated estrogens (104.6%) were in excellent agreement with the stated values. The results (84.30%) for total quantities of sodium equilin sulphate and sodium estrone sulphate (Table 2) and their ratio (0.511) obtained by the proposed fluorodensitometric method for tablets meet the requirements of USP XXI for Conjugated Estrogens Tablets [18]. The results obtained suggest that the proposed method may be suitable for the assay of conjugated estrogens in raw materials or tablets.

Table 2
Assay of tablets containing oestrogens (1.25 mg)

Compound	Found*	Percentage†	RSD %
Sodium estrone sulphate	0.692	55.74	7.03
Sodium equilin sulphate	0.354	28.53	3.61
Sodium 17-α-dihydroequilin sulphate	0.075	6.08	5.42
Sodium 17-α-estradiol sulphate	0.066	5.12	2.62
Sodium equilenin sulphate	0.028	2.30	2.21
Sodium 17-α-dihydroequilenin sulphate	0.024	1.91	7.79
Total amount	1.240	99.20	

*Sodium sulphate esters of estrogens expressed in milligrams per tablet.
†Average of seven determinations.

References

[1] R. E. Schirmer, in *Modern Methods of Pharmaceutical Analysis*, pp. 237–239. CRC Press, Florida (1986).
[2] K. A. Connors, in *A Textbook of Pharmaceutical Analysis*, pp. 235–247. Wiley-Interscience (1982).
[3] T. James, *J. Pharm. Sci.* **59**, 1648–16449 (1970).
[4] T. James, *J. AOAC* **56**, 86–87 (1973).
[5] J. Bramhall and A. Z. Britten, *Clin. Chim. Acta* **68**, 203–213 (1976).
[6] P. M. Karba, F. H. Tsai and L. J. Marton, *Clin. Chim. Acta* **128**, 9–17 (1983).
[7] S. Fishman, *J. Pharm. Sci.* **64**, 674–679 (1975).
[8] L. P. Penzes and G. W. Oertel, *J. Chromatogr.* **51**, 325–327 (1970).
[9] L. P. Penzes and G. W. Oertel, *J. Chromatogr.* **74**, 359–365 (1972).
[10] R. W. Roos, *J. Pharm. Sci.* **67**, 1735–1739 (1978).
[11] R. W. Roos and C. A. Lau-Cam, *J. Pharm. Sci.* **74**, 201–204 (1985).
[12] G. J. Krol, R. P. Masserano, J. F. Carney and B. T. Kho, *J. Pharm. Sci.* **59**, 1483–1487 (1970).
[13] W. Eechaute, G. Demeester and I. Leusen, *Steroids* **13**, 101–119 (1969).
[14] J. E. Hammond, J. C. Phillips and J. Savory, *Clin. Chem.* **24**, 631–634 (1978).
[15] T. James, *J. Pharm. Sci.* **61**, 1306–1308 (1972).
[16] C. Dumazert, C. Ghiglione and T. Pugnet, *Ann. Pharm. Fr.* **21**, 227–232 (1963).
[17] S. Udenfriend, in *Fluorescence Assay in Biology and Medicine*, pp. 349–371. Academic Press, London (1964).
[18] *The United States Pharmacopeia*, 21st rev., pp. 401–402, Convention, Rockville, MD (1985).

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