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Densitometric determination of conjugated oestrogens in the raw material and in pharmaceutical preparations

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Abstract: A densitometric method for determination of complex mixtures of conjugated oestrogens in raw material and tablets was developed. The proposed procedure comprised hydrolysis of sodium sulphate esters of oestrone, equilin, 17α-oestradiol, equilenin, 17α-dihydroequilin and 17α-dihydroequilenin, the chloroform extraction of free oestrogens and methyltestosterone (internal standard) and separation on TLC plates using chloroform–cyclohexane–dioxane–triethylamine (4.5:4.1:0.6, v/v) as the solvent for development. Quantitative assay was achieved by direct scanning of the oestrogen spots at 280 nm. The proposed method is simple, rapid, reproducible and adequate to control the content of conjugated oestrogens in the raw material and in pharmaceutical preparations.

Keywords: *Conjugated oestrogens; TLC separation; densitometry; quantitative assay; raw material; sample preparation.*

Introduction

"Conjugated oestrogens" described in the USP XXI [1] is a mixture of sodium oestrone sulphate and sodium equilin sulphate, derived wholly or in part from equine urine, or synthetically from oestrone and equilin. It may contain other conjugated oestrogenic substances of the type excreted by pregnant mares. They are widely used for the treatment of certain hormone deficiencies. Many methods, including colorimetric-iron-Kober reaction [2, 3], paper chromatography [4], liquid chromatography (LC) [5], thin-layer chromatography (TLC) [6–9], gas chromatography (GC) [10–14] and high-performance liquid chromatography (HPLC) [15] have been used to separate and in some cases quantitate the components of this relatively complex mixture. Most of these methods involve enzyme or acid hydrolysis of sulphate esters, extraction of free oestrogens, formation of derivatives (such as trimethylsilyl, metoxamine-trimethylsilyl, oxime-trimethylsilyl esters, dansyl or ABS) and finally separation using TLC, GC or HPLC. In recent years, reversed-phase and silver-impregnated HPLC have been used to separate the oestrogen sulphates, while ion-pair HPLC has been used to separate both oestrogen sulphates and free oestrogens [16]. TLC separation has been achieved for the identification of oestrogens on TLC plates impregnated with silver nitrate [17, 18] or formamide [19] using dual [20] or multiple [9] elution systems. However, a quantitative TLC method that allows for the direct determination of equine oestrogens has not been previously reported.

The present paper will focus on the development of a simple, accurate and rapid procedure for the direct densitometric determination of conjugated oestrogens in the raw material and in preparations.

Experimental

Materials

Oestrone, equilin, equilenin, 17α-oestradiol and methyltestosterone were purchased from the Sigma Chemical Co. 17α-Dihydroequilin and 17α-dihydroequilenin were obtained from Diosynth (Oss, Holland). The TLC plates (20 × 20 cm precoated with 0.25 mm silica gel $60F_{254}$) were purchased from Merck (Darmstadt, FRG). A Hamilton microlitre syringe was used for applying solutions. All solvents were of analytical grade. Raw material of conjugated oestrogens "Diosynth" (Oss, Holland) contained 604 mg per g oestrone, equilin, equilenin and their 17-hydroxy deriv-

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atives. Hormopleks® sugar-coated tablets (Galenika, Zemun, Yugoslavia) each contained 1.25 mg of conjugated oestrogens.

Standard solutions

Stock solutions of 0.8 mg ml^{-1} oestrone and 0.4 mg ml^{-1} equilin, equilenin, 17α-dihydroequilin and 17α-dihydroequilenin were prepared in benzene. The stock solution of 0.4 mg ml^{-1} 17α-oestradiol was prepared in benzene–ethanol (4:1, v/v). Standard solutions of oestrone and equilin were prepared by transferring appropriate volumes of the corresponding solutions to 5-ml volumetric flasks with the addition of 1 ml of 1.5 mg ml^{-1} methyltestosterone solution and then diluting to volume with benzene. 3 μl of each standard solution was applied to TLC plates to give a series of spots in a range of 0.1–2 μg oestrone or 0.08–1.5 μg equilin, in order to evaluate the linearity of graphs of peak–height ratios of oestrogens relative to methyltestosterone, the internal standard. Standard solutions of equilenin, 17α-dihydroequilenin, 17α-dihydroequilin and 17α-oestradiol were prepared by transferring appropriate volumes of the corresponding solutions to 5-ml volumetric flasks with the addition of 0.4 ml of 1.5 mg ml^{-1} methyltestosterone solution and then diluting to volume with benzene. 15 μl of each standard solution was applied to the TLC plates to give a series of spots in a range 0.060–1.2 μg equilenin and 17α-dihydroequilenin or 0.080–2 μg 17α-oestradiol and 17α-dihydroequilin.

Preparation of sample extracts

Raw material. A quantity of raw material containing about 25 mg of conjugated oestrogens was placed in a beaker and dissolved in 20 ml of methanol. 20 ml of water, 4 ml of concentrated hydrochloric acid and several boiling clips were added, and the mixture was boiled for 5 min. After cooling to room temperature, 5 ml of a 1.5 ml mg^{-1} methyltestosterone solution in methanol was transferred into a separating funnel and extracted with 2 × 10-ml and 1 × 5-ml portions of chloroform. The combined extract was washed with 5 ml water, passed through 1 g of anhydrous sodium sulphate into a 25-ml volumetric flask and diluted to volume with chloroform. 3 μl of extract was applied to the TLC plates for quantitative assay of oestrone and equilin. The same procedure was performed with 1 ml of 1.5 mg ml^{-1} methyltestosterone solution. Application of 15 μl of extract permits the quantitative assay of 17α-oestradiol, equilenin, 17α-dihydroequilin and 17α-dihydroequilenin.

Tablets. The sugar-coated layer of conjugated oestrogen tablets was carefully removed with water and filter dried. Thirty tablets were weighed and powdered. A quantity of powder containing about 25 mg of conjugated oestrogens was extracted with 70 ml of methanol and filtered through a quantitative filter into a beaker. 70 ml of water and 4 ml of concentrated hydrochloric acid were added. Further steps were as described for the raw material.

Spectrodensitometry

Spots of solutions of the sample extract and appropriate standard solutions of oestrogens were applied to TLC plates. The plates were developed in a saturated tank using chloroform–cyclohexane–dioxane–triethylamine (45:40:10:6, v/v) as solvent. The solvent front was allowed to migrate for about 18 cm. The plate was air-dried and the spots were detected under UV light at 254 nm. The chromatographic zones corresponding to the spots of oestrogens and methyltestosterone on the TLC plates were scanned at 280 nm with a Camag–TLC–Scanner using the reflectance mode, under the following scanning conditions: slit length 6 mm, slit width 1.2 mm, scan speed 1 mm s^{-1}, recorder voltage 20 mV and chart speed 50 mm min^{-1}.

Results and Discussion

Densitograms of six equine oestrogens and methyltestosterone as internal standard, extracted from raw material, separated and scanned at 280 nm, are shown in Fig. 1. The R_f values of oestrone, equilin, 17α-oestradiol, equilenin, 17α-dihydroequilin, 17α-dihydroequilenin and methyltestosterone were 0.58, 0.51, 0.44, 0.39, 0.30, 0.25 and 0.74, respectively. The peak–height ratio of each oestrogen relative to methyltestosterone was measured and the content of conjugated oestrogens calculated using the equation:

$$R_u/R_s \times C_s \times Q/Q_x \times T \times 1.38,$$

where R_u = peak–height ratio of each oestrogen relative to methyltestosterone either from the sample extract of raw material or from

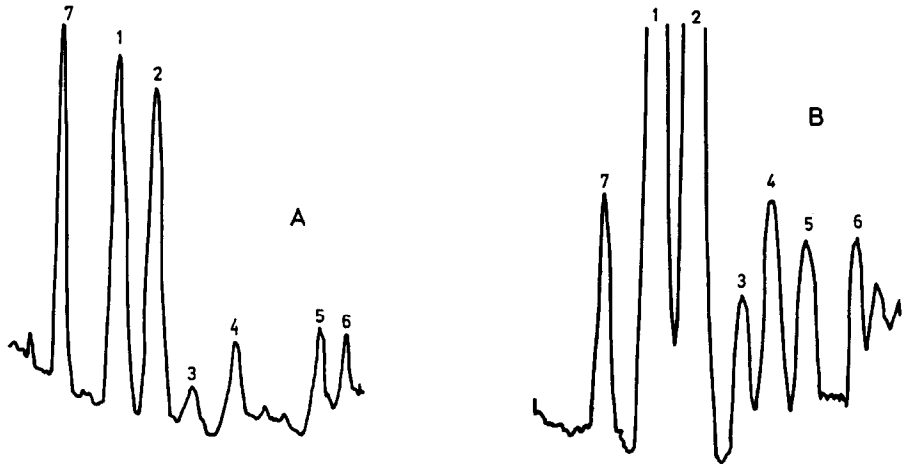

Figure 1
Densitograms of oestrone (1), equilin (2), 17α-oestradiol (3), equilenin (4), 17α-dihydroequilin (5), 17α-dihydroequilenin (6) and methyltestosterone (7) after the application of 3 μl (A) or 15 μl (B) sample extract, obtained by scanning at 280 nm.

Table 1
Raw material assay

Compound	Declared*	Found*	Percentage†	RSD‡ (%)	Recovery (%)
Sodium oestrone sulphate	334.0	340.5	58.0	5.3	101.9
Sodium equilin sulphate	180.0	159.1	27.1	4.9	88.4
Sodium 17-dihydroequilin sulphate	35.8	32.6	5.6	3.1	91.1
Sodium 17-dihydroequilenin sulphate	8.6	8.9	1.5	3.8	103.9
Sodium equilenin sulphate	16.1	15.3	2.6	4.6	94.9
Sodium 17-oestradiol sulphate	29.6	28.9	5.0	1.9	106.6
Total amount	604.0	586.2	—	—	97.1

*The content of sodium sulphate esters of oestrogens expressed as mg per g of raw material.
†Average of 10 determinations.
‡RSD = relative standard deviation.

Table 2
Assay of 1.25-mg tablets

Compound	Found*	Percentage†	RSD (%)
Sodium oestrone sulphate	0.740	57.0	3.4
Sodium equilin sulphate	0.370	28.5	5.8
Sodium 17-dihydroequilin sulphate	0.076	5.8	2.9
Sodium 17-dihydroequilenin sulphate	0.018	1.4	3.7
Sodium equilenin sulphate	0.027	2.1	3.4
Sodium 17-oestradiol sulphate	0.058	4.5	2.9
Total amount	1.298	—	—

*The content of sodium sulphate esters of oestrogens expressed as mg per tablet.
†Mean of 10 determinations.

tablets; R_s = peak–height ratio of each standard of oestrogen relative to methyltestosterone; C_s = the concentration of standard solutions of oestrogens applied to the plates in mg per ml; Q = the amount of either the raw material or tablet powder declared to contain 25 mg of conjugated oestrogens; Q_x = the weighed amount of either raw material or tablet powder used in the assay; T = label claimed amount of conjugated oestrogens in either the raw material or coated tablets; and 1.38 is a factor converting free oestrogens to the conjugated sodium salts. Results of the determination of conjugated oestrogens from the raw material and coated tablets are shown in Tables 1 and 2. *In situ* maximum absorption

by measuring diffuse reflectance was established for oestrone, equilin, 17α-dihydroequilin, 17α-dihydroequilenin and methyltestosterone at 280 nm, and 17α-oestradiol and equilenin at 290 nm. The recorded spectra of equal amounts of methyltestosterone, 17α-oestradiol, oestrone and equilenin are presented in Fig. 2. A good correlation between amounts of each oestrogen applied to the plate and their peak–height ratios relative to methyltestosterone were achieved ($r \geq 0.993$). The correlation coefficient (r), regression equations, standard error (S_{xy}) and y-axis intercept error (Sa) with statistical significance (P) were: oestrone $r = 0.995$, $y = 0.105 + 1.199x$, $S_{xy} = 0.061$, $Sa = 0.087$, $P > 0.2$;

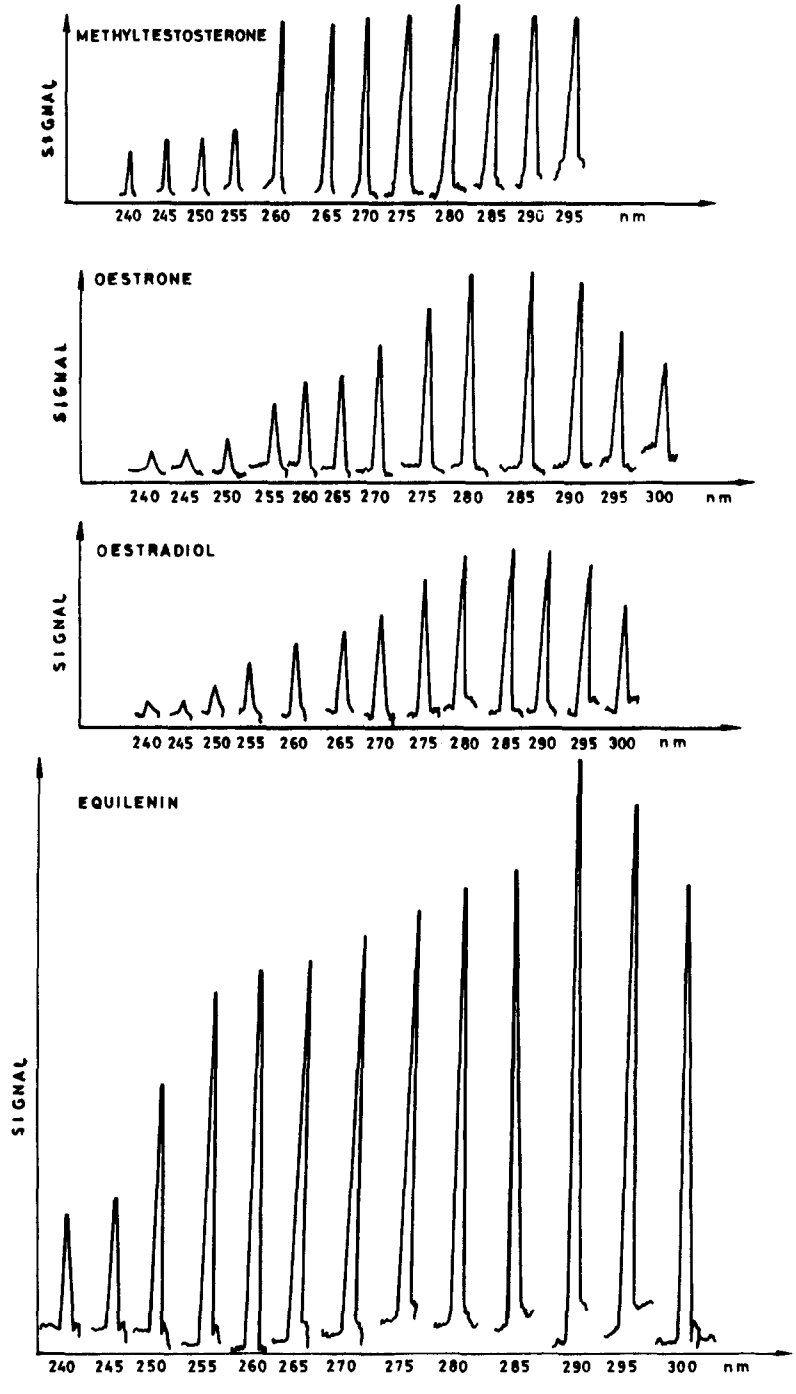

Figure 2
Spectra of equal amounts of the same substance as in Fig. 1 recorded in a TLC Scanner (Camag, Switzerland).

equilin $r = 0.993$, $y = -0.047 + 2.35x$, $S_{xy} = 0.074$, $Sa = 0.069$, $P > 0.5$; 17α-oestradiol $r = 0.997$, $y = 0.003 + 1.09x$, $S_{xy} = 0.024$, $Sa = 0.017$, $P > 0.8$; equilenin $r = 0.996$, $y = -0.041 + 5.87x$, $S_{xy} = 0.15$, $Sa = 0.145$, $P > 0.7$; 17α-dihydroequilin $r = 0.996$, $y = 0.05 + 1.41x$, $S_{xy} = 0.047$, $Sa = 0.05$, $P > 0.3$; and 17α-dihydroequilenin $r = 0.995$, $y = 0.046 + 3.953x$, $S_{xy} = 0.075$, $Sa = 0.048$, $P > 0.3$. In all cases $P \geqslant 0.2$ is evidence for the reliability of the method. The proposed procedures for the densitometric determination of conjugated oestrogens from both the raw material and tablets included methanol extraction and acid hydrolysis. Here the acid–methanol hydrolysis of oestrogen sulphate esters was performed similarly to the method of Caragnan and Lodge (1976). Johnson *et al.* (1975), McErlane and Curran (1977) and USP XXI alike suggested enzyme hydrolysis. However, the results of Carignan and Lodge (1980) indicated that there was no difference between enzyme and acid hydrolysis employed for sample preparation. Chloroform extraction of free oestrogens followed and the quantitation had to be done within 48 h after extraction. Application to the plates and separation of structurally similar oestrogens using a single elution system developed in this study allowed for the quantitative assay to be performed by scanning at 280 nm. In earlier reports, the TLC separation of this mixture of oestrogens was achieved using multiple and continuous [20] elution systems or TLC plates impregnated with silver nitrate and formamide [17–19], respectively. The separation of oestrogens by single development as accomplished here, requires a shorter interval as compared with that of reference data for dual or continual elution development of this mixture. Separation on unimpregnated TLC plates $F60_{254}$ provided an opportunity for UV detection for the purpose of identification and determination.

Under the given scanning conditions the lower detection limit for all oestrogens in the mixture was 0.060 μg. In regard to the label declaration, the recovery values obtained for each oestrogen determined in the raw material and the total potency of conjugated oestrogens (97.1%) or tablets (103.2%), were very high. Values of the relative standard deviations obtained for each oestrogen from raw material (RSD $\leqslant 5.3\%$) or for tablets of conjugated oestrogens (RSD $\leqslant 5.8\%$) confirm the accuracy and reproducibility of the method. The quantities of equilin and oestrone and their ratio (0.51) are within the limits specified in the USP XXI for the raw material and tablets. The proposed micro method of direct densitometric determination of conjugated oestrogens is sensitive, simple, rapid and may be adequate for the control of the content of raw material and preparations.

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