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Short Communication

High-performance liquid chromatographic determination of equine estrogens with ultraviolet absorbance and electrochemical detection

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Abstract

The simultaneous separation of a mixture of equine estrogens containing estrone, equilin, equilenin and their corresponding 17α-diols was accomplished by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Nucleosil C_{18} column with a mobile phase composed of methanol–water–2-propanol–dichloromethane (45:42.5:7.5:5, v/v). UV absorbance and electrochemical detection were used for the analysis of a standard mixture of equine estrogens. RP-HPLC with UV absorbance detection was applied to the determination of these compounds in pharmaceutical drug substances and drug products after acid hydrolysis of their sodium sulfate esters.

1. Introduction

Conjugated estrogens are defined in the USP XXII [1] as a mixture of sodium estrone sulfate and sodium equilin sulfate obtained synthetically or isolated from equine urine. They may contain other pharmacologically active conjugated estrogenic substances, such as equilenin, 17α-estradiol, 17α-dihydroequilin and 17α-dihydroequilenin, and traces of the corresponding 17β-diols.

The official USP XXII method [1] for the determination of conjugated estrogens is based on the GC of trimethylsilyl ethers of estrone and equilin after enzymatic hydrolysis of their sodium sulfate esters. Recently, capillary GC [2] and densitometry [3,4] of underivatized equine estrogens in drug substances and drug products have been reported. HPLC has been described for the determination of the estrogens after hydrolysis of the conjugates and formation of dansyl derivatives [5]. RP-HPLC of conjugated forms of equine estrogens was proposed by Flann and Lodge [6], but the quantitative evaluation of the method was not reported. Townsend et al. [7] developed an RP-HPLC determination of conjugated and free forms of estrone and equilin with UV absorbance detection. The separation of nine equine estrogens by RP-HPLC has been reported by Desta [8].

This paper describes an RP-HPLC method with UV absorbance and electrochemical detec-

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tion (ED) for the determination of estrone (ES), equilin (EQ), equilenin (EQN), 17α-estradiol, 17α-dihydroequilin (DHEQ) and 17α-dihydroequilenin (DHEQN) after acid hydrolysis of their conjugates. This method was applied to pharmaceutical drug substances and drug products.

2. Experimental

2.1. Apparatus and operating conditions

The HPLC measurements with UV absorbance detection were carried out by using a Chrompack (Middelburg, Netherlands) HPLC Gras system equipped with CP UV-VAR detector and an injection valve with a 20-μl loop (Chrompack), interfaced with an IBM PC/2 Model 30 computer, connected to an Epson LQ 550 recorder (Seico Epson, Japan) and a Chrompack BD 70 recorder (Kipp and Zonen, Delft, Netherlands). The mobile phase consisted of methanol–water–2 - propanol–dichloromethane (45:42.5:7.5:5, v/v). The flow-rate was 0.7 ml/min and UV absorption detection was performed at 280 nm.

The HPLC–ED measurements were carried out by using an LC-XPD pump (Pye Unicam) equipped with an injection valve with a 3-μl loop and an ADLC 2 amperometric detector (Laboratorní Přístroje, Prague, Czech Republic) connected with a TZ 4620 line recorder (Laboratorní Přístroje). Ag–AgCl was used as the reference electrode, carbon fibre as the working electrode and stainless steel as the counter electrode. $Na_2HPO_4 \cdot 12H_2O$ (0.6%) was added to the above mobile phase, which was adjusted to pH 6.0–6.05 with acetic acid.

All separations were performed on a Nucleosil C_{18} stainless-steel column (250 × 4.6 mm I.D., 3-μm particle size) (Chrompack).

2.2. Materials

ES, EQ, EQN and estriol [internal standard (I.S.)] were obtained from Sigma (St. Louis, MO, USA) and ESD, DHEQ and DHEQN were gifts from Diosynth (Oss, Netherlands). Drug substance containing 604 mg/g of conjugated estrogens was obtained from Diosynth, Hormopleks sugar-coated tablets, each containing 1.25 mg of conjugated estrogens, from Galenika (Zemun, Yugoslavia) and Oestro-Feminal capsules, each containing 1.25 mg of conjugated estrogens, from Heinrich Mach (Illertisen, Germany).

2.3. Calibration plots

Stock standard solutions of ES, EQ, EQN, ESD, DHEQ, DHEQN and the I.S. were prepared at a concentration of 0.5 mg/ml in methanol and stored at 4°C. Working standard solutions for obtaining calibration graphs were prepared by transferring 1 ml of I.S. stock standard solution into 10-ml volumetric flasks and diluting to the mark with methanol. The final concentrations of estrogens in the working standard solutions for calibration were 0.005, 0.01, 0.02, 0.05, 0.1, 0.15, 0.2 and 0.3 mg/ml.

2.4. Sample preparation

Acid hydrolysis of conjugated estrogens and extraction of the 3-phenol forms of equine estrogens from drugs were carried out as described previously [4]. A 2.5-ml volume of I.S. stock standard solution (0.5 mg/ml in methanol) was added to the hydrolysate previously cooled to room temperature. The estrogens were extracted with 2 × 10 and 1 × 5 ml of chloroform and the combined extracts were washed with 5 ml of water, passed through 1 g of anhydrous sodium sulfate and evaporated to dryness under a nitrogen stream and the residue was dissolved in 25 ml of methanol–water (1:1).

3. Results and discussion

According to the results of Carignan and Lodge [9] and Clairns et al. [10], there is no significant difference between acid and enzymatic hydrolysis of conjugated estrogens. The separation of all compounds was achieved within less than 25 min and the retention times of the I.S., DHEQN, DHEQ, ESD, EQN, EQ and ES

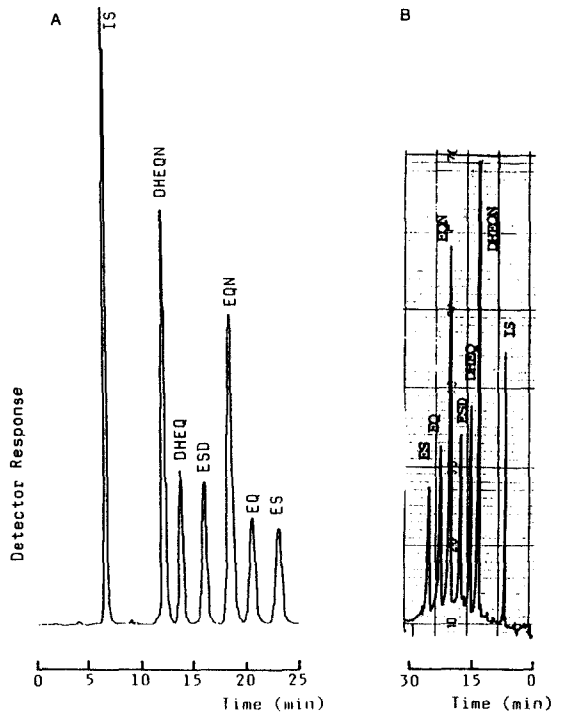

Fig. 1. Typical chromatograms of the standard calibration mixture of equine estrogens containing 0.05 mg/ml each of the I.S., DHEQN, DHEQ, ESD, EQN, EQ and ES obtained by (A) HPLC–UV detection and (B) HPLC–ED. Chromatographic conditions: (A) column, Nucleosil C_{18} (250 × 4.6 mm I.D.), mobile phase, methanol–water–2-propanol–dichloromethane (45:42.5:7.5:5, v/v); flow-rate, 0.7 ml/min; loop injection (20 μl); UV absorbance detection at 280 nm; (B) mobile phase, as above but containing 0.6% of $Na_2HPO_4 \cdot 12H_2O$; loop injection (3 μl); applied potential, 1.1 V; 100 nA full-scale; other conditions as in (A).

were 6.44, 11.85, 13.36, 15.14, 18.59, 20.59 and 23.18 min, respectively. Details of the separation will be presented in a separate paper [11]. ED requires the use of a conducting mobile phase, which is why 0.06% of $Na_2HPO_4 \cdot 12H_2O$ was added to the mobile phase.

Fig. 1 represents typical chromatograms of a calibration mixture containing 0.05 mg/ml of the I.S., DHEQN, DHEQ, ESD, EQN, EQ and ES obtained by (A) RP-HPLC with UV absorbance detection and (B) RP-HPLC with ED detection. The potential of the electrochemical detector was set at 1.1 V vs. Ag–AgCl. On increasing the potential, the detector response showed a plateau.

Statistical evaluation of the calibration data obtained by UV absorbance and electrochemical detection is given in Tables 1 and 2, respectively.

Estriol meets all the requirements for an ideal I.S. [12], as its UV absorption maximum (280 nm) and electrochemical properties are similar to those of the analyte compounds. As ED depends critically on numerous experimental conditions [12], RP-HPLC–UV detection was applied to the determination of equine estrogens in drugs. The better selectivity of ED might be of benefit in the determination of these compounds in biological materials. HPLC–ED has been used successfully for the determination of catechol estrogens [13,14] and estriol glucuronides [15] in biological fluids. By this means the tedious clean-up procedure was avoided [15]. To our knowledge, the

Table 1
Statistical evaluation of the calibration data obtained by RP-HPLC with UV absorbance detection

Compound	$y = a + bx$[a]	r[b]	Range of linearity[c]	LOD[d]
DHEQN	$y = -0.0534 + 1.1266x$	0.9994	0.005–0.3	0.002
DHEQ	$y = -0.0460 + 0.4736x$	0.9988	0.005–0.3	0.004
ESD	$y = -0.0363 + 0.4986x$	0.9982	0.005–0.3	0.004
EQN	$y = -0.0859 + 1.3526x$	0.9993	0.005–0.3	0.002
EQ	$y = -0.0389 + 1.4757x$	0.9989	0.010–0.3	0.004
ES	$y = -0.0529 + 0.4868x$	0.9985	0.010–0.3	0.004

[a] y = Relative detector response (estrogen/I.S. peak-area ratio); x = concentration of the estrogen in the calibration solution divided by I.S. concentration; a = intercept on the ordinate; b = slope.
[b] r = Correlation coefficient.
[c] In mg/ml, which corresponds to injected amounts from 0.1 ng DHEQN, DHEQ, ESD, EQN (0.2 ng EQ, ES) to 6 ng in 20 μl.
[d] Limit of detection (mg/ml) at a signal-to-noise ratio of 3.

Table 2
Statistical evaluation of the calibration data obtained by RP-HPLC with ED

Compound	$y = a + bx$[a]	r[b]	Range of linearity[c]	LOD[d]
DHEQN	$y = 0.1783 + 1.4641x$	0.9960	0.005–0.05	0.002
DHEQ	$y = 0.0147 + 0.7241x$	0.9992	0.005–0.05	0.004
ESD	$y = 0.0178 + 0.5921x$	0.9995	0.005–0.05	0.004
EQN	$y = 0.1437 + 1.2096x$	0.9969	0.005–0.05	0.002
EQ	$y = 0.0726 + 0.4704x$	0.9965	0.010–0.05	0.004
ES	$y = 0.0452 + 0.3907x$	0.9986	0.010–0.05	0.004

[a] y = Relative detector response (estrogen/I.S. peak-height ratio); x = concentration of the estrogen in the calibration solution divided by I.S. concentration; a = intercept on the ordinate; b = slope.
[b] r = Correlation coefficient.
[c] In mg/ml, which corresponds to injected amounts from 0.015 ng DHEQN, DHEQ, ESD, EQN (0.03 ng EQ, ES) to 0.15 ng in 3 μl.
[d] Limit of detection (mg/ml) at a signal-to-noise ratio of 3.

Table 3
Determination of equine estrogens by RP-HPLC–UV absorbance detection in drugs

Compound	Drug substance			Hormopleks tablets			Oestro-Feminal capsules		
	D[a] (%)	F[b] (%)	R.S.D.[c] (%)	D[a] (%)	F[b] (%)	R.S.D.[c] (%)	D[a] (%)	F[b] (%)	R.S.D.[c] (%)
ES	55.29	56.76	1.54	–[d]	54.93	0.99	70–90	82.36	1.57
EQ	29.80	27.69	5.21	–	26.17	1.64	8–15	8.39	1.94
EQN	2.66	4.28	6.19	–	4.79	4.25	≤4	2.09	5.37
ESD	5.93	5.42	6.00	–	5.76	3.44		2.22	6.11
DHEQ	4.90	3.85	5.92	–	6.17	5.54	2–8[e]	2.29	8.13
DHEQN	1.42	1.86	3.73	–	2.32	2.51		1.48	6.24

[a] Declared amount.
[b] Found amount.
[c] Relative standard deviation of ten determinations.
[d] Not specified.
[e] Total amount of ESD, DHEQ and DHEQN.

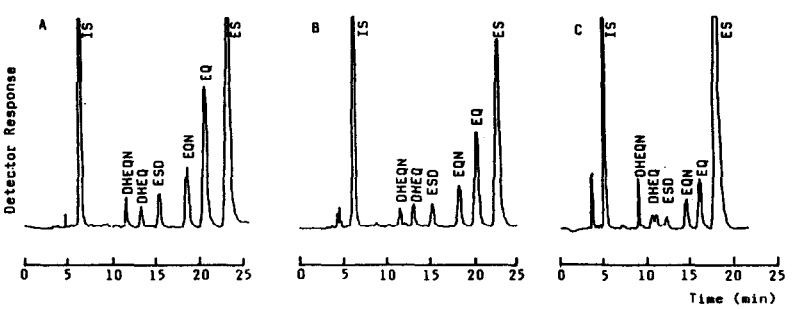

Fig. 2. Typical chromatograms of (A) the drug substance, (B) Hormopleks tablets and (C) Oestro-Feminal capsules. Chromatographic conditions as in Fig. 1A.

determination of equine estrogens by HPLC–ED detection has not previously been reported.

The results of the determination of equine estrogens in drugs by HPLC–UV detection are given in Table 3 and the chromatograms are presented in Fig. 2. The results imply that the HPLC–UV detection method can replace the official USP XXII assay for the determination of conjugated estrogens in drugs.

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