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HPLC analysis of pharmaceutical estrogens in raw materials and dosage forms

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Abstract

The use of HPLC with fluorescence detection ($\lambda_{ex} = 280$ nm; $\lambda_{em} = 410$ or 312 nm) in combination with a postcolumn on line photochemical derivatization was investigated for the analysis of conjugated and unconjugated estrogens and their correlated impurities. The column effluents were subjected on-line to UV irradiation (254 nm) and the photo induced modifications were useful for the identification of the various estrogens. The proposed HPLC methods were successfully applied to the analysis of commercially available conjugated estrogens (raw materials and pharmaceuticals) and estrogen samples. The assay results relative to the pharmaceutical formulations were in agreement with those obtained by a reference HPLC method with UV detection ($\lambda = 280$ nm). © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Reversed-phase liquid chromatography; Conjugated estrogens; Photochemical derivatization; Fluorescence detection

1. Introduction

Coniugated estrogens (USP XXIII) is a mixture of sodium estrogen-3-sulphates, derived totally or in part from equine urine or from synthetic mixtures, primarily of sodium estrone sulphate (52.5–61.5%) and sodium equilin sulphate (22.5–30.5%). They may contain other conjugated estrogenic substances of the type excreted by pregnant mares, such as 17α-dihydroequilin, equilenin, 17α-dihydroequilenin, 17α-estradiol and traces of the corresponding 17β-diols [1]. They are of considerable importance in hormone replacement therapy, for prevention of osteoporosis, cardiovascular desease in postmenopausal women and in some selected case of breast and prostatic cancers [2].

The official method (USP XXIII) for the determination of conjugated and esterified estrogens involve a laborious gas chromatographic (GC) procedure after enzymatic hydrolysis. For the tablets, the USP method is based on liquid chromatography (HPLC) with UV detection ($\lambda = 210$ nm) and requires only that the ratio of sodium estrone sulphate to sodium equilin sulphate must fall in the range 0.35–0.65%. Various methods have been described for the analysis of conjugated estrogen with and without acid or enzimatic hy-

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drolysis; these include capillary gas chromatography [3,4], HPLC with UV [5–10], electrochemical [7] and fluorimetric [11,12,17] detection, supercritical fluid chromatography (SFC) [13], densitometry [14,15], and capillary electrophoresis [16]. Fluorescence detection was applied after dansyl derivatization [11,12]. The native fluorescence of some estrogens was also utilized; in particular, HPLC-fluorescence was applied to the determination of 17β-estradiol in serum [17] and the fluorodensitometric determination of equilenin and 17α-dihydroequilenin in pharmaceuticals was used [14].

The aim of this work was to developed simple, rapid and sensitive HPLC procedures (fluorescence and UV detection) for the analysis of conjugated estrogens without hydrolysis and at providing information-rich detection to achieve unambiguous identification of the various components. To this end, fluorescence detection was used in combination with postcolumn on-line photochemical derivatization. Using a photoreactor arranged online between the column and the detector, the column effluent was subjected to UV irradiation (254 nm) and photo-induced alterations resulted in modified spectral properties of the analytes. Using fluorescence and ultraviolet detection the HPLC method was successfully applied to the analysis of commercial formulations of conjugated estrogens.

The HPLC procedure with fluorimetric detection proved to be also suitable for the quality control of estrogen raw materials (sodium estrone sulphate, sodium equilin sulphate, estrone and equilin) allowing the sensitive determination of selected impurities.

2. Experimental

2.1. Materials

Sodium equilin sulphate (EQ sulphate), sodium equilenin sulphate (EQN sulphate), sodium 17α-dihydroequilin sulphate (17α-DHEQ sulphate), sodium 17α-dihydroequilenin sulphate (17α-DHEQN sulphate), 17α- and 17β-dihydroequilin (17α- and 17β-DHEQ) and 17α- and 17β-dihydroequilenin (17α- and 17β-DHEQN) were obtained from Wyeth-Ayerst Research (Rouses Point, NY 12979, USA). Sodium estrone sulphate (ES sulphate), estrone (ES), equilin (EQ) were from Sigma (St. Louis, MO, USA), equilenin (EQN) from Aldrich Chimica (Italy), hexadecyltrimethylammonium bromide (CTAB) and 3,5-dimethylbenzoic acid from Fluka (Buchs, Switzerland). Triethylamine (TEA) and the other chemicals were from Carlo Erba Reagent (Italy). The TEA phosphate buffer (pH 3.0 or 4.0; 0.05 M) were prepared adding ortophosphoric acid to an aqueous TEA solution up to the desired pH. Organic solvents for chromatography were HPLC grade (Mallinkrodt, USA) and double distilled water was used.

2.2. Apparatus

The liquid chromatograph comprised a Varian 2010 pump, a Varian 2070 fluorescence spectrophotometer and Jasco FP-920 fluorescence spectrophotometer, operating at an emission wavelength at 410 and 312 nm with an excitation wavelength of 280 nm, connected to a personal computer AcerView 34TL. The integration program JCL6000 was used. A second liquid chromatograph comprised Waters 501 pump and Jasco Uvidec-100-V spectrophotometer, operating at 280 nm, connected to a HP 3396 integrator. 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). Emission and excitation spectra were recorded on a Perkin-Elmer LS-3 spectrofluorimeter using 1 cm quartz cells. 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 $\times$ 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. UV spectra were recorded on a Jasco Uvidec 610 double-beam spectrophotometer using 1 cm cells. Sonarex Super RK 102 (35 KMz) Bandelin (Berlin, Germany) equipment with termostatically controlled heating (30–80°C) was used for ultrasonication.

Table 1
Data for calibration graphs ($n=6$) of conjugated and unconjugated estrogens

Drug	Method[a]	Slope[b]	y-Intercept	Correlation coefficient	Concentration range[c]
ES sulphate	Fl	0.0144	−0.0037	0.9999	75.45–503.00
	UV	1.3367	0.0895	0.9980	0.01–0.70
EQ sulphate	F1	0.0993	−0.0168	0.9993	21.33–160.10
	UV	1.1069	0.1640	0.9980	0.03–0.70
17α-DHEQ sulphate	Fl	0.1055	0.0209	0.9978	9.37–156.25
	UV	2.2891	0.0795	0.9990	0.01–0.30
17α-DHEQN sulphate	Fl	2.6400	−0.0048	0.9979	0.24–7.98
EQN sulphate	Fl	1.0190	0.0379	0.9987	0.83–5.55
	UV	5.7644	−0.0524	0.9990	0.002–0.10
EQN sulphate	Fl	0.4402	0.1336	0.9982	2.50–10.10
EQN	Fl	1.7870	−0.3809	0.9993	0.73–4.38
ES	Fl	0.0691	0.0364	0.9997	13.17–158.10
EQ	Fl	2.6180	0.3130	0.9986	0.80–5.62

[a] F1, HPLC-fluorimetric method; UV, HPLC-UV method.
[b] Peak-height of the drug (h) for HPLC-fluorimetric method; ratio of analyte peak-height to internal standard peak-height (h_a/h_s) for HPLC-UV method.
[c] Concentration is measured in μg ml^{-1} (Fl) and mg ml^{-1} (UV).

The routine chromatographic separations were performed on a Hypersil 5 ODS (250 × 4.6 mm i.d.), Phenomenex Prodigy 5 ODS_2 (250 × 4.6 mm i.d.) and Phenomenex Phenosphere 5 BDS (250 × 4.6 mm i.d.) under isocratic conditions.

2.3. Cromatographic conditions

2.3.1. Analysis of impurities

The quality control of estrogens (raw materials) was carried out using the HPLC fluorimetric method. Routine analyses were performed on a 250 × 4.6 mm i.d. column packed with Hypersil 5 ODS. Sodium equilin sulphate was chromatographed using a ternary mixture of 10 mM CTAB buffer (pH 3.0)/ethanol/tetrahydrofuran (52:38:10, v/v/v) as mobile phase at a flow rate of 1.0 ml min^{-1}; sodium estrone sulphate was analysed using a mixture A/B (62:38, v/v), where A is TEA phosphate buffer (pH 3.0; 0.05 M) and B is ethanol/tetrahydrofuran (70:30, v/v) at a flow rate of 1.0 ml min^{-1}; equilin and estrone using as mobile phase the mixture A/B (73:27, v/v), where A is TEA phosphate buffer (pH 3.0; 0.05 M) and B is ethanol/tetrahydrofuran (70:30, v/v) at a flow rate of 1.3 ml min^{-1}.

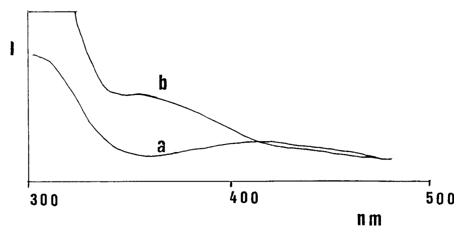

Fig. 1. Emission spectra of EQ ($\lambda_{em}=$ 300-500 nm; bandwidth = 18 nm) with on line photoreactor switched (a) off and (b) on. Temperature = 35°C. Column: Phenomenex Phenosphere 5 ODS (250 × 4.6 mm i.d.). Mobile phase: TEA phosphate buffer (pH 4.0; 0.05 M)/acetonitrile (64:36, v/v) at a flow rate of 1 ml min^{-1}. I, fluorescence intensity.

2.3.2. Analysis of dosage forms

2.3.2.1. Fluorimetric method. The HPLC separation of the coniugated estrogens with fluorimetric detection was carried out at 35 ± 2°C using a Phenomenex Prodigy column 5 ODS_2 (250 × 4.6 mm i.d.). The mobile phase was TEA phosphate buffer (pH 4.0; 0.05 M)/acetonitrile (70:30, v/v) at a flow rate of 1.0 ml min^{-1}. For unconjugated estrogens a mobile phase of TEA phosphate buffer (pH 4.0; 0.05 M)/acetonitrile (66:34, v/v) at flow rate of 1.3 ml min^{-1} was used.

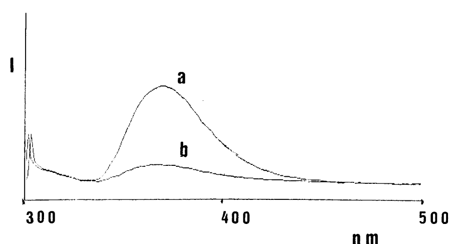

Fig. 2. Emission spectra of EQN (λ_{em} = 300-500 nm; bandwidth = 18 nm) with on line photoreactor switched (a) off and (b) on. Temperarure = 35°C. Chromatographic conditions as in Fig. 1. I, fluorescence intensity.

2.3.2.2. UV method. The HPLC separation of the conjugated estrogens with UV detection was performed using a Phenomenex Phenosphere 5BDS column (250 × 4.6 mm i.d.) with a mobile phase of TEA phosphate buffer (pH 4.0; 0.05 M)/acetonitrile (70:30, v/v) at a flow rate of 1.0 ml min^{-1}.

2.4. Calibration graphs

Stock solutions of the estrogens were prepared in the mobile phase and were stored for 1 week at 4°C.

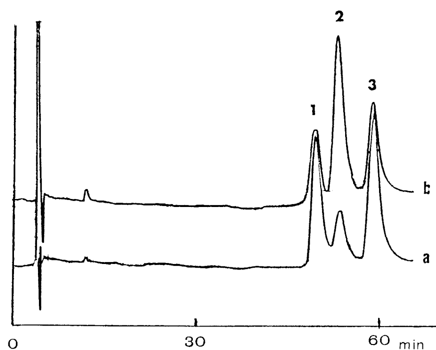

Fig. 3. HPLC chromatogram at 35°C of an estrone real bulk sample and its relative impurities with on-line photoreactor switched (a) off and (b) on. Peaks: 1, EQN; 2, EQ; 3, ES. Column: Hypersil 5 ODS (250 × 4.6 mm i.d.). Mobile phase: mixture A/B (73:27, v/v), where A is TEA phosphate buffer (pH 3.0; 0.05 M) and B is ethanol/tetrahydrofuran (70:30, v/v) at a flow rate of 1.3 ml min^{-1}. Fluorescence detection: λ_{ex} = 280 nm; λ_{em} = 410 nm.

For the analyses of estrogens at impurity level, standard solutions of EQN sulphate (2.50–10.10 μg ml^{-1}), ES (13.17–158.10 μg ml^{-1}), EQ (0.80–5.62 μg ml^{-1}) and EQN (0.73–4.38 μg ml^{-1}) were prepared in the mobile phase and injected in triplicate into the chromatograph; the peak height was plotted against the analyte concentration to obtain the calibration graphs.

For the analysis of dosage forms both fluorescence and UV detections were applied to obtain the calibration graphs (concentration ranges in Table 1). When UV detection was used the standard solutions contained 3,5-dimethylbenzoic acid (0.70 mg ml^{-1}) as the internal standard.

2.5. Analysis of raw materials

Selected impurities were determined in samples of EQ sulphate, ES sulphate, EQ and ES, by a direct method (method A) and the standard addition method (method B).

Method A

- EQ sulphate: a sample of about 6.00 mg was dissolved in 10 ml of the mobile phase.
- ES sulphate: a sample of about 15 mg was dissolved in 2 ml of ethanol/water (50:50, v/v).
- EQ: a sample of about 6 mg of EQ was dissolved in 5 ml of ethanol and then diluted with the mobile phase to obtain about 240 mg ml^{-1}.
- ES: a sample of about 7 mg of ES was dissolved in 5 ml of ethanol and then diluted with the mobile phase to obtain about 718 mg ml^{-1}.

The impurity content in each samples was determined by comparison with an appropriate standard solution.

In method B the standard addition method was applied. A fixed volume of the impurity solution at increasing concentration was added to the sample solution (method A) in the same solvent.

Method B

- EQ sulphate: 1 ml aliquots of standard solution of EQN sulphate (2.50–10.10 μg ml^{-1}) dissolved in ethanol/water (50:50, v/v) were added to 1.0 ml of EQ sulphate (0.60 mg ml^{-1}); 1.0 ml aliquots of standard solution of EQ (1.66–3.33 μg ml^{-1}) in the mobile phase were added to 1.0 ml of EQ sulphate (0.60 mg ml^{-1}).

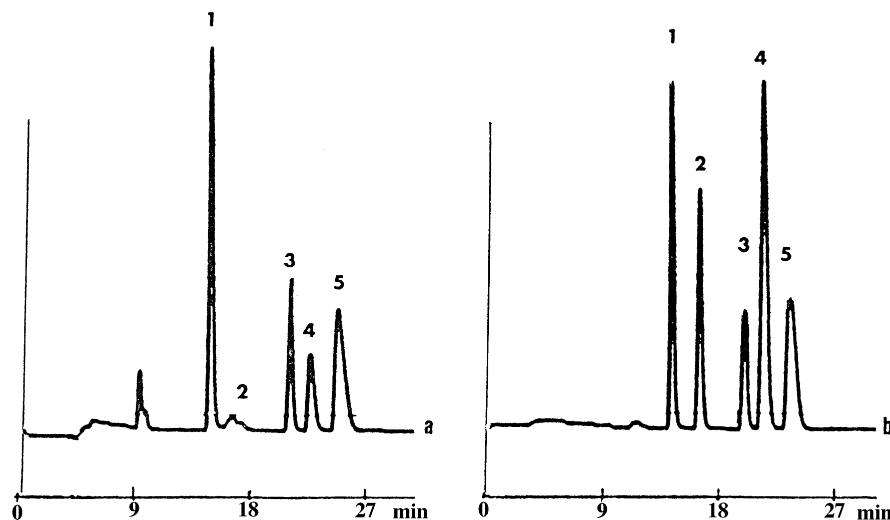

Fig. 4. Representative HPLC separation at 35°C of the conjugated estrogens with on-line photoreactor switched (a) off and (b) on. Peaks: 1, 17α-DHEQN sulphate; 2, 17α-DHEQ sulphate; 3, EQN sulphate; 4, EQ sulphate; 5, ES sulphate. Column: Phenomenex Prodigy 5 ODS (250 × 4.6 mm i.d.). Mobile phase: TEA phosphate buffer (pH 4.0; 0.05 M)/acetonitrile (70:30, v/v) at a flow rate of 1.0 ml min^{-1}. Detection as in Fig. 3.

- ES sulphate: 0.3 ml aliquots of ES solution (13.17–158.10 μg ml^{-1}) in the mobile phase were added to 0.3 ml of ES sulphate (7.70 mg ml^{-1}).
- EQ: 0.5 ml aliquots of EQN solution (0.73–4.38 μg ml^{-1}) in the mobile phase were added to 0.5 ml of EQ (0.24 mg ml^{-1}).
- ES: 0.3 ml of EQ solution (1.60–8.03 μg ml^{-1}) and 0.3 ml of EQN solution (0.076–0.38 μg ml^{-1}) in the mobile phase were added to 0.3 ml of ES (0.718 mg ml^{-1}).

The peak-height of analyte (EQN sulphate, ES, EQN and EQ) were plotted against the corresponding amounts added to obtain for each analyte a standard addition calibration graph. The x-intercept was then used for calculating the content of impurity (EQN sulphate, ES, EQN and EQ) in the samples analysed.

2.6. Analysis of pharmaceutical formulations

2.6.1. Sample preparation

2.6.1.1. Lyophilized. An amount of commercial sample corresponding to 0.22 mg of conjugated estrogens was dissolved, by ultrasonication for 1 min, in 5 ml of mobile phase, containing 0.07 mg ml^{-1} of the internal standard for the HPLC-UV method. The resulting solution was filtered with 0.45 μm nylon 4 mm filter and used for the HPLC analyses.

2.6.1.2. Tablets. An amount of powdered tablet equivalent to about of 0.32 mg conjugated estrogens was treated, by ultrasonication for 10 min, with 10 ml of mobile phase, containing 0.007 mg ml^{-1} of the internal standard for HPLC-UV method. The resulting suspension was filtered with 0.45 μm nylon 4 mm filter and then subjected to the HPLC analyses.

3. Results and discussion

The first objective of this study was the development of a practical HPLC method of the estrogen impurities determination in raw material and of the conjugated estrogens in commercial dosage forms. An additional aim was the unambiguous identification of the mixture components by the combination of the fluorescence detection with postcolumn on-line photochemical derivatization

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 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. For qualitative information, the emission spectra of EQ and EQN are reported in Fig. 1 and Fig. 2, obtained with on-line photoreactor switched off (a) and on (b). After photochemical derivatization, the spectra undergo significant reverse modifications.

3.1. Chromatography and detection

Fig. 3 shows the chromatogram obtained from a ES sample (raw material) containing EQ and EQN as impurities. The estrogens were shown to be highly photosensitive and the chromatograms obtained at $\lambda_{em} = 410$ nm with $\lambda_{ex} = 280$ nm by the reactor switched off (a) and on (b) display significant changes in the peak intensity. In particular, EQ fluorescence intensity increases, while EQN and ES fluorescence intensity decreases. The 410 nm wavelenght was chosen as a compromise suitable for the simultaneous detection of the various estrogens present in very different concentrations.

Table 2
Relative standard deviations of estrogen impurities and conjugated estrogens obtained from replicate ($n = 8$) analyses of a single standard solution

Compound	Concentration (mg ml^{-1})	R.S.D. %
EQN sulphate	3.00	1.9
EQN	2.90	1.7
ES	43.00	2.3
EQ	2.40	2.6
17α-DHEQN sulphate	2.45	0.7
17α-DHEQ sulphate	56.63	2.3
EQ sulphate	32.18	2.7
ES sulphate	99.32	0.6

The favourable effect of the on-line UV irradiation of the eluate on the fluorescence intensity was confirmed for some conjugated estrogens. A representative separation of the conjugated estrogens ($\lambda_{em} = 410$ nm with $\lambda_{ex} = 280$ nm) with the photoreactor switched off (a) and on (b) is illustrated in Fig. 4. As it can be seen, 17α-DHEQ sulphate and EQ sulphate fluorescence intensity increases significantly by irradiation at 254 nm. In the Fig. 5 the chromatograms of unconjugated estrogens are showed using $\lambda_{em} = 312$ nm, with $\lambda_{ex} = 280$ nm. These separations can be used for the analyses of conjugated estrogens after enzymatic or chemical hydrolysis. Particularly, considerable enhancement of the EQ and ES fluorescence intensity at $\lambda_{em} = 312$ nm with $\lambda_{ex} = 280$ nm can be achieved. Significant interference from the main Raman band of water was not observed according to the literature data [17]. The reduced bandwidth (18 nm) of the used spectrofluorimetric detector also allowed to avoid this problem, which may occur using filter fluorimetric detectors.

Preliminary investigations have been performed on the photo-induced modifications of the equi-

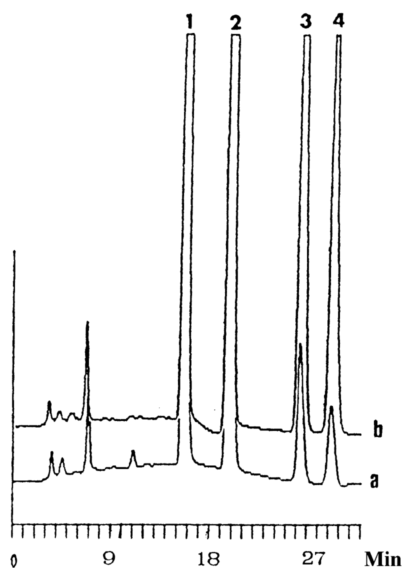

Fig. 5. Representative HPLC separation at 35°C of unconjugated estrogens with on-line photoreactor switched (a) off and (b) on. Peaks: 1, 17β-DHEQ; 2, 17α-DHEQ; 3, EQ; 4, ES. Column as in Fig. 4. Mobile phase: TEA phosphate buffer (pH 4.0; 0.05 M)/acetonitrile (66:34, v/v) at a flow rate of 1.3 ml min^{-1}. Fluorescence detection: $\lambda_{ex} = 280$ nm; $\lambda_{em} = 312$ nm.

Table 3
Correlation coefficients obtained by the standard addition method for the determination of selected impurities in commercial estrogen samples

Compound	Impurity	Correlation coefficient	Conc. range impurity added (μg ml^{-1})
EQ sulphate	EQ	0.9994	1.66–3.33
	EQN sulphate	0.9992	2.5–10.10
EQ	EQN	0.9998	0.73–4.38
ES sulphate	ES	0.9996	13.17–158.10
ES	EQ	0.9993	1.60–8.03
	EQN	0.9980	0.076–0.38

line chromophore responsible for the enhancement of the fluorescence. Chromatographic and fluorescence data suggest a partial conversion of equiline to equilenin under irradiation at 254 nm. Further studies are in progress for the confirmation.

3.2. Analysis of the impurities

Under the described chromatographic conditions linear relationships between peak-height and analyte concentration were found for these impurities (Table 1). The precision of the methods was satisfactory as indicated by the relative standard deviations obtained from replicate ($n = 8$) analyses of a single standard solution (Table 2). Moreover, in order to validate the method suitability (fluorimetric detection) the impurities were also quantified by the standard addition method, by adding increasing known amounts of pure standards to the original estrogen (ES sulphate, EQ sulphate, ES and EQ) solutions. The amount of a given impurity was calculated by using the x-intercept of a plot of peak-height (y) versus estrogen impurity added (x). The obtained correlation coefficients and the impurities concentration ranges for the analysis of commercial samples are reported in Table 3. The results obtained for commercial samples of ES sulphate, ES, EQ and EQ sulphate are reported in Table 4. As can be seen, the individual content of EQN and EQ in ES sample can be selectively determined by the proposed HPLC-fluorescence method which, therefore, appears to be advantageous over the official colorimetric procedure (USP XXIII).

3.3. Analysis of pharmaceutical formulations

Linear relationships between the peak-height (y) and drug concentration (x) using both the detection methods (HPLC-fluorescence and HPLC-UV) were obtained (Table 1). The satisfactory within-run precision of the method was indicated by the relative standard deviation of the peak-height from replicate ($n = 8$) analyses of a single standard solution (Table 2).

The detection limit (signal to noise ratio = 3) is reported in Table 5. The method with fluorimetric detection, after photochemical derivatization, is about 1000-times more sensitive than UV detection. The methods were then applied to HPLC analyses of commercial formulations of conjugated estrogens, using fluorimetric detection at $\lambda_{em} = 410$ nm with $\lambda_{ex} = 280$ nm and UV detection at $\lambda = 280$ nm. The assay results for each formulation were found to be in good agreement with the declared content (Table 6). The results obtained using fluorescence detection were comparable with those obtained with UV detection. Chromatograms obtained from commercial samples are illustrated in Fig. 6 and Fig. 7. The accuracy of the methods was verified by analysing samples fortified by known quantities of the drug; essentially quantitative recoveries (98.8–99.7%) were obtained. These data support the suitability of the methods for the quality control of conjugated estrogens dosage forms.

Table 4
Results from HPLC analysis with fluorimetric detector of estrogens as impurities in raw materials.

Compound	Impurity	% Found (R.S.D. %) (method A)[a]	% Found (R.S.D. %) (method B)[b]	Detection limit ($S/N=3$) % impurity/sample	λ_{ex}	λ_{em}
ES sulphate	ES	0.130 (3.3)	0.170 (3.9)	0.0003	280	410
EQ sulphate	EQN sulphate	0.470 (3.0)	0.510 (2.8)	0.0025	280	410
	EQ[c]	0.080 (4.5)	0.090 (4.1)	0.0013	280	312
ES	EQ[c]	0.260 (3.5)	0.320 (3.1)	0.0400	280	410
	EQN	0.009 (3.4)	0.010 (3.2)	0.0030	280	410
EQ	EQN	0.680 (3.4)	0.730 (2.9)	0.0030	280	410

[a] Method A, reference method; the results are the average of four determinations and expressed as a percentage of the claimed content.
[b] Method B, standard addition method.
[c] Postcolumn photochemical derivatization was applied.

Table 5
Detection limit of conjugated estrogens (S/N = 3)

Compound	HPLC-fluorimetric method (pmol)			HPLC-UV method (nmol)
	Photoreactor (on/off)	$\lambda_{ex} = 280$ nm $\lambda_{em} = 312$ nm	$\lambda_{em} = 410$ nm	$\lambda = 280$ nm
ES sulphate	on	0.19	1.38	1
EQ sulphate	on	0.08	0.33	1
17α-DHEQ sulphate	on	0.03	0.32	1
17α-DHEQN sulphate	off	—	0.04	—
EQN sulphate	off	—	0.01	0.01

4. Conclusion

The proposed HPLC-fluorimetric method has considerable potential as pharmacopeial method for the assay of natural mixtures of estrogen sulphates, quality control of their formulations, and for monitoring the free steroids. The combination of fluorimetric detection with postcolumn on-line photoderivatization constitutes a simple and effective approach to enhancing the sensitivity and the selectivity of an HPLC method, and offers the opportunity to achieve further information useful for the reliable quality control of estrogen in raw materials and conjugated estrogens in dosage forms. The described direct HPLC-UV procedure, developed as useful reference method, proved to be a convenient analytical method suitable for the determination of coniu-

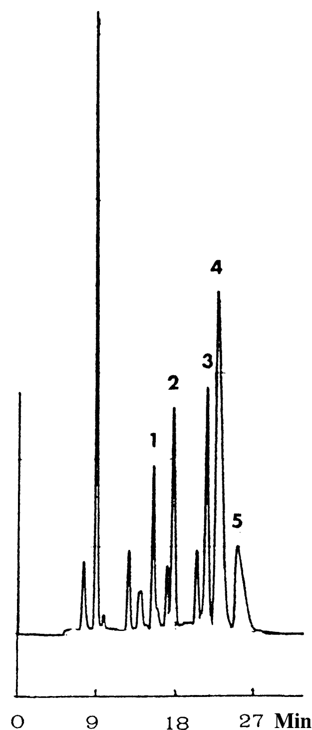

Fig. 6. HPLC chromatogram at 35°C of conjugated estrogens (lyophilized). Peaks: 1, 17α-DHEQN sulphate; 2, 17α-DHEQ sulphate; 3, EQN sulphate; 4, EQ sulphate; 5, ES sulphate. Chromatographic conditions as in Fig. 4. Detection as in Fig. 3.

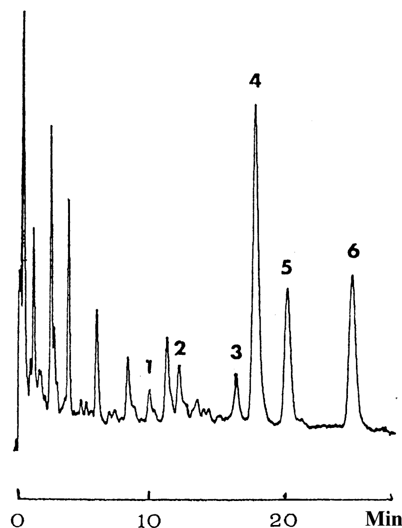

Fig. 7. HPLC chromatogram of conjugated estrogens (tablets). Peaks: 1, 17α-DHEQN sulphate; 2, 17α-DHEQ; 3, EQN sulphate; 4, EQ sulphate; 5, ES sulphate; 6, Internal standard (3,5-dimethylbenzoic acid). Column as in Fig. 1. Mobile phase as in Fig. 4. UV detection: $\lambda = 280$ nm.

Table 6
Results by HPLC analysis of coniugated estrogens in commercial pharmaceutical formulations

Formulation[a]	Drug	Found[b] HPLC-fluorimetric. method	R.S.D. %	Found[b] HPLC-UV method	R.S.D. %	Ratio EQ/ES	
						HPLC-fluor. method	HPLC-UV method
Lyophilized	ES	55.45	1.8	57.16	2.5	0.47	0.49
	EQ	26.05	1.2	28.03	3.0	—	—
	17α-DHEQ	14.50	2.3	13.50	2.8	—	—
	17α-DHEQN	0.25	1.5	—	—	—	—
	EQN	1.18	1.9	1.90	2.9	—	—
Tablets	ES	56.70	1.9	57.00	2.4	0.44	0.47
	EQ	25.08	2.2	27.03	3.1	—	—
	17α-DHEQ	14.43	1.6	13.67	3.0	—	—
	17α-DHEQN	0.40	1.8	—	—	—	—
	EQN	1.92	2.1	1.38	2.2	—	—

[a] Other ingredients. Lyophilized: lactose, sodium citrate, simethicone. Tablets: lactose, methylcellulose, magnesium stearate, shellac powder, polyethylenglycol, glycerilmonooleate, calcium sulphate anhydrous, saccharose, microcrystalline cellulose, colouring agent E110, colouring agent E104, carnauba wax, titanium dioxide, stearic acid.

[b] Mean of five determinations and expressed as a percentage of the claimed content.

gated estrogens in pharmaceutical formulations. Studies are in progress for the application of the HPLC-fluorescence method, in combination with on-line photochemical derivatization, to the analysis of biological samples because the photochemical derivatization offers a remarkable sensitivity for EQ, 17α- and 17β-DHEQ and ES.

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References

[1] United States Pharmacopeia XXIII and The National Formulary XVIII, Pharmacopeial Convention, INC, Rockville, MD, USA, 1995, pp. 627–631.
[2] C.L. Williams, G.M. Stancel, The pharmacological basis of therapeutics, in: Goodman and Gilman's (Eds.), 9th ed., McGraw-Hill, NY, 1995, pp. 1411–1424.
[3] G.W. Lyman, R.N. Johnson, J. Chromatogr. 234 (1982) 234–239.
[4] J. Novakovic, E. Tvrzicka, J. High Resolut. Chromatogr. 14 (1991) 495–498.
[5] G. Capitano, R. Tochezne, J. Pharm. Sci. 68 (1979) 311–313.
[6] R.W. Roos, J. Assoc. Off. Anal. Chem. 63 (1980) 80–87.
[7] J. Novakovic, E. Tvrzicka, V. Pacakova, J. Chromatogr. 678 (1994) 359–363.
[8] B. Flann, B. Lodge, J. Chromatogr. 402 (1987) 273–282.
[9] R. W Townsend, U. Keuth, K. Embil, G. Mullersman, J.H. Perrin, H. Derendurt, J. Chromatogr. 450 (1988) 414–419.
[10] B. Desta, J. Chromatogr. 435 (1988) 385–390.
[11] R.W. Roos, T. Medwik, J. Chromatogr. Sci. 18 (1980) 626–630.
[12] R.W. Roos, C.A. Lau-Cam, J. Pharm. Sci. 74 (1985) 201.
[13] N.K. Jagota, J.T. Stewart, J. Pharm. Biomed. Anal. 10 (1992) 667–673.
[14] J. Novakovic, D. Agbaba, D. Zivanov Stakic, J. Pharm Biomed. Anal. 7 (1989) 1657–1662.
[15] S.K. Poole, M.T. Belay, C.F. Poole, J. Planar Chromatogr. Mod. TLC 5 (1992) 16–27.
[16] J.C. Touchstone, S.S. Levin, J. Liq. Chromatogr. 17 (1994) 3925–3931.
[17] S.Y. Su, G.K. Shiu, J. Simmons, C.T. Viswanathan, J.P. Skelly, Biomed. Chromatogr. 6 (1992) 265–268.