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ORIGINAL ARTICLE

Determination of 17 β-estradiol in pharmaceutical preparation by UV spectrophotometry and high performance liquid chromatography methods

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Abstract In this study, new, rapid UV spectrophotometry (UV) and reversed phase high performance liquid chromatography (HPLC) methods were developed for the determination of 17 β-estradiol in pure and in pharmaceutical dosage form. The solvent system, wavelength of detection and chromatographic conditions were optimized in order to maximize the sensitivity of both the proposed methods. The linear regression equations obtained by least square regression method were $y = 0.0184x + 0.0059$ for the UV method and $y = 56742x - 3403.6$ for the HPLC method. The developed methods were successfully employed with a high degree of precision and accuracy for the estimation of total drug content in a commercial tablet of 17 β-estradiol. The results obtained from the UV method were compared with those obtained by using HPLC. The proposed methods are highly sensitive, precise and accurate and can be used for the reliable quantitation of 17 β-estradiol in pharmaceutical dosage form.

1. Introduction

17 β-estradiol (Fig. 1) is the most potent of the natural human estrogens (Russell et al., 2000). It is the most potent estrogen of a group of endogenous estrogen steroids which includes estrone and estriol.

17 β-estradiol is responsible for the growth of breast and reproductive epithelia, maturation of long bones and development of secondary sexual characteristics. 17 β-estradiol and its semi-synthetic esters are primarily used as menopausal hormones. It may also be used as replacement therapy for female hypogonadism or primary ovarian failure. The decrease of 17 β-estradiol at menopause is often accompanied by vascular instability and rise in incidence of heart disease and an increasing risk of osteoporosis (Havlikova et al., 2006).

Several analytical methods for the qualitative and quantitative determination of 17 β-estradiol have been described, such as voltammetry (Salci and Biryol, 2002), high performance liquid chromatography (HPLC) (Russell et al., 2000; Lamparczyk et al., 1994; Yamada et al., 2002; Terada et al., 1992; Mao et al., 2003; Nygaard et al., 2004; Yilmaz and Kadioglu, 2013), liquid

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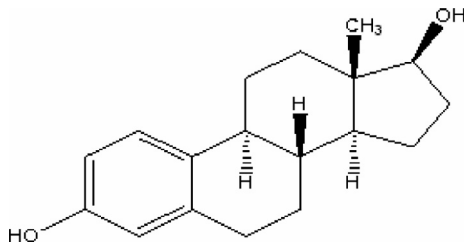

Figure 1 Chemical structure of 17 β-estradiol.

chromatography-tandem mass spectrometry (LC–MS–MS) (Ingrand et al., 2003) and gas chromatography–mass spectrophotometry (GC–MS) (Zacharia et al., 2004; Fotsis and Adlercreutz, 1987; Adlercreutz et al., 1975; Gaskeiland Brownsey, 1983; Adlercreutz et al., 1974; Castagnetta et al., 1992).

On an extensive survey of the literature, no UV spectrophotometric method is reported till date for the determination of 17 β-estradiol in pure form and in pharmaceutical dosage form. However, survey of the literature revealed two HPLC methods for the determination of 17 β-estradiol in pharmaceutical formulations. HPLC method for 17 β-estradiol and 17 β-estradiol-3-acetate using C_{18} column and mobile phase of acetonitrile–water (50:50, v/v) has been reported (Russell et al., 2000). The other one is the reverse phase HPLC method (Havlikova et al., 2006) which utilized acetonitrile-0.085% phosphoric acid-tetrahydrofurane (27:63:10, v/v/v) as mobile phase system for 17 β-estradiol from its solid dosage form. Over the last 10 years, a HPLC method using electrochemical detection technique has been reported for the determination of 17 β-estradiol in plasma (Yamada et al., 2002).

USP 1995 has recommended HPLC method for the analysis of pure 17 β-estradiol and in dosage form (tablet). The method recommended the use of a mobile phase of acetonitrile–water (55:45, v/v) at a flow rate of 1 mL min^{-1}.

In the present study, two simple, economical, accurate and reproducible analytical methods were developed for the determination of 17 β-estradiol in pure form and in its tablet form.

The UV method was aimed at developing an easy and rapid assay method for 17 β-estradiol without any time consuming sample preparation steps for routine analysis, to be adopted in quality control and drug testing laboratories, and at the same time ensure satisfactory recovery during drug determination from pharmaceutical formulation.

HPLC method was attempted to demonstrate the utility of fluorescence detection for the determination of 17 β-estradiol coupled with simple and economical mobile phase and reasonable analysis time with high precision.

In both the proposed methods, there is no need to extract the drug from the formulation excipient matrix thereby decreasing the error in quantization. Formulation samples can be directly used after dissolving and filtration. The developed methods were used to determine the total drug content in commercially available pharmaceutical preparation of 17 β-estradiol.

2. Experimental

2.1. Chemicals

17 β-estradiol (99.6% purity) was purchased from Sigma (St. Louis, Mo, USA). HPLC grade methanol (99.8% purity) was purchased from Fluka (Buchs, Switzerland). High quality pure water was prepared using Millipore purification system (Millipore, Molsheim, France). Estrofem tablet containing 2 mg 17 β-estradiol was obtained from pharmacy (Erzurum, Turkey).

2.2. Equipments

Thermospectronic double beam UV–Vis spectrophotometer (HEλIOSβ, Thermospectronic, Cambridge, UK) with the local control software was used. UV spectra of the solutions were recorded in 1 cm quartz cells at a scan speed of 600 nm min^{-1}.

The HPLC system consisted of a Thermoquest Spectra System P 1500 isocratic pump coupled with a Spectra System UV 6000 LP fluorescence detection system, a Spectra System AS 3000 autosampler, a SCM 1000 vacuum membrane degasser, and a SN 4000 system controller. The detector was set to scan from 200 to 500 nm and had a discrete channel set at detection (excitation at 280 nm and emission at 310 nm), which was the wavelength used for quantification.

2.3. Chromatographic conditions

The chromatographic column used was a reversed phase 4.6 mm × 250 mm Phenomenex C_{18} column (Merck, Darmstadt, Germany) with 5 μm particles. The column and the HPLC system were kept in ambient conditions. The mobile phase was methanol–water (70:30, v/v) prepared at a flow rate of 1.0 mL min^{-1}. The injection volume was 20 μL.

2.4. Preparation of standard curve for the UV method

A stock solution of 17 β-estradiol was prepared by dissolving 10 mg of drug in 100 mL of methanol. The λ_{max} of 17 β-estradiol in methanol was determined by scanning a suitable dilution of the stock using the UV–Vis spectrophotometer. From the stock solution, various standard dilutions were made to obtain solutions of 0.5, 1, 1.5, 2, 4, 6, 8, 10 and 12 μg mL^{-1} and their respective absorbance was measured.

2.5. Preparation of standard curve for the HPLC method

A stock solution (100 μg mL^{-1}) of pure drug was prepared by dissolving 5 mg 17 β-estradiol in 50 mL of methanol. From this stock solution, 50, 125, 250, 500, 750, 1500, 2000, 4000, 5000 and 6000 ng mL^{-1} standard solutions were prepared by suitable dilution in 10 mL volumetric flask.

2.6. Procedure for pharmaceutical preparation

A total 10 tablets of 17 β-estradiol (Estrofem tablet) were accurately weighed and powdered. An amount of this powder corresponding to one tablet of 17 β-estradiol content was weighed and accurately transferred into 100 mL calibrated flask and 75 mL of methanol was added and then the flask was sonicated for 10 min at room temperature. The flask was filled to volume with methanol. The resulting solutions in both the cases were filtered through Whatman filter paper no 42 and suitably diluted to get a final concentration within the limits of linearity for the respective proposed methods. The drug content of 17 β-

estradiol tablet was calculated from the absorbance value (UV method) and the peak area (HPLC method).

2.7. Data analysis

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 10.0. Correlations were considered statistically significant if calculated P values were 0.05 or less.

3. Results

3.1. Validation of the methods

To evaluate the validation of the present methods, parameters such as specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), recovery, specificity and stability were investigated according to the ICH validation guidelines (ICH, 1996).

3.2. Linearity

The linearity range of 17 β-estradiol solution in case of the UV method was found to be 0.5–12 μg mL^{-1}. The linearity range of the HPLC method was obtained as 50–6000 ng mL^{-1}. The calibration curves constructed were evaluated by their correlation coefficients. The calibration equations from six replicate experiments demonstrated the linearity of the methods. Standard deviations of the slope and intercept for the calibration curves are given in Table 1.

3.3. Precision and accuracy

The precision of the UV and HPLC methods was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by analyzing quality control samples six times per day, at three different concentrations which were quality control samples. The intermediate precision was evaluated by analyzing the same samples twice daily for three days. The accuracy of this analytical method was assessed as the percentage relative error. For all the concentrations studied, intra- and inter-day RSD values were $\leqslant$3.86% and relative errors were $\leqslant$4.70%. These results are given in Table 2.

3.4. Limit of detection (LOD) and quantification (LOQ)

For spectrophotometry measurements, LOD and LOQ of 17 β-estradiol were determined using calibration standards. The LOD and LOQ values were calculated as 3.3 σ/S and 10 σ/S, respectively, where S is the slope of the calibration curve and σ is the standard deviation of y-intercept of regression equation ($n = 6$).

For HPLC measurements, the LOD and LOQ of 17 β-estradiol were determined by injecting progressively low concentrations of the standard solution under the chromatographic conditions. The lowest concentrations assayed where the signal/noise ratio was at least 10:1, this concentration was regarded as LOQ. The LOD was defined as a signal/noise ratio of 3:1. The LOD and LOQ for spectrophotometry were 0.15 and 0.45 μg mL^{-1}, for HPLC 10 and 25 ng mL^{-1}, respectively. Among the two methods, HPLC is more sensitive than spectrophotometry (Table 1).

3.5. Stability

To evaluate the stability of 17 β-estradiol, standard solutions were prepared separately at concentrations covering the low, medium, and higher ranges of calibration curves for different temperatures and times. These solutions were stored at room temperature, refrigerated (4 °C) and frozen (−20 °C) for 24 h and 48 h. The accuracy of 17 β-estradiol stability obtained for the room temperature, 4 and 20 °C refrigeration temperatures was 99.8, 98.5 and 99.5%, respectively. These are within the acceptance range of 90–110%. The results are given in Table 3.

3.6. Specificity

The specificity of the two methods was investigated by observing any interference encountered from the common tablet excipients, such as talc, gelatin, lactose, corn starch, magnesium stearate, methyl hydroxypropyl cellulose, indigotin and titanium dioxide. These excipients did not interfere with the proposed methods.

3.7. Recovery

To determine the recovery of the UV and HPLC methods and to study the interference of formulation additives, the recovery

Table 1 Linearity of 17 β-estradiol.

Parameter	Spectrophotometry	HPLC
Linearity	0.5–12 (μg mL^{-1})	50–6000 (ng mL^{-1})
Regression equation[a]	$y = 0.0184x + 0.0059$	$y = 56742x - 3403.6$
Standard deviation of slope	2.58×10^{-3}	601.7
Standard deviation of intercept	8.36×10^{-4}	0.6387
Correlation coefficient	0.9956	0.9993
Standard deviation of correlation coefficient	3.25×10^{-3}	2.52×10^{-4}
Limit of detection (ng mL^{-1})	150	10
Limit of quantification (ng mL^{-1})	450	25

[a] Based on six calibration curves, y = absorbance (UV method) and peak area (HPLC method) x = concentration of 17 β-estradiol in μg mL^{-1} (UV method) and in ng mL^{-1} (HPLC method).

Table 2 Precision and accuracy of 17 β-estradiol.

Added	Intra-day			Inter-day		
	Found ± SD[a]	Precision % RSD[b]	Accuracy[c]	Found ± SD[a]	Precision % RSD[b]	Accuracy[c]
Spectrophotometry (μg mL^{-1})						
3	3.08 ± 0.037	1.20	2.67	3.12 ± 0.054	1.73	4.00
5	4.98 ± 0.034	0.68	−0.40	5.02 ± 0.069	1.37	0.40
7	7.01 ± 0.034	0.49	0.14	7.03 ± 0.143	2.04	0.43
HPLC (ng mL^{-1})						
100	97.90 ± 2.809	2.87	−2.10	98.40 ± 3.089	3.14	−1.60
1000	1029 ± 28.400	2.76	2.90	1047 ± 40.414	3.86	4.70
4500	4473 ± 53.229	1.19	−0.60	4586 ± 109.147	2.38	1.91

[a] SD: Standard deviation of six replicate determinations.
[b] RSD: Relative standard deviation.
[c] Accuracy: % relative error: (found-added)/added × 100.

Table 3 Stability of 17 β-estradiol in solution (n = 6).

Intra-day				Inter-day	
Method	Room temperature 24 h	Refrigeratory 4 °C, 24 h	Frozen −20 °C, 24 h	Refrigeratory 4 °C, 48 h	Frozen −20 °C, 48 h
Spectrophotometry (μg mL^{-1})					
0.5	99.8 ± 1.35	97.6 ± 2.59	99.3 ± 1.77	99.3 ± 2.16	99.1 ± 3.26
6	99.6 ± 0.46	99.3 ± 3.17	99.7 ± 1.17	99.5 ± 1.41	99.8 ± 2.19
12	100.1 ± 0.35	98.7 ± 2.87	100.3 ± 2.16	100.4 ± 2.56	100.6 ± 2.39
HPLC (ng mL^{-1})					
100	97.6 ± 4.12	98.1 ± 3.85	99.4 ± 3.12	97.7 ± 4.97	99.6 ± 3.78
2000	98.5 ± 2.47	99.2 ± 2.27	99.7 ± 2.08	98.6 ± 3.06	100.7 ± 3.15
6000	101.2 ± 2.71	101.7 ± 2.34	100.6 ± 1.89	102.1 ± 3.54	101.8 ± 2.76

was checked as three different concentration levels. Analytical recovery experiments were performed by adding known amount of pure drugs to pre-analyzed samples of commercial dosage form. The percent analytical recovery values were calculated by comparing concentrations obtained from the spiked samples with actual added concentrations. These values are also listed in Table 4.

Five sets of experiments for this drug were carried out using two different analysts; no significant difference was obtained between the results in this study. The developed methods were applied to the determination of 17 β-estradiol in Estrofem tablet. According to the statistical comparison (Student's t-test) of the results there is no significant difference between UV and HPLC methods (Table 5).

3.8. System suitability

A system suitability test of the chromatography system was performed before each validation run. Five replicate injections of a system suitability/calibration standard and one injection of a check standard were made. Area relative standard deviation, tailing factor, and efficiency for the five suitability injections were determined. The check standard was quantified against the average of the five suitability injections. For all

Table 4 Recovery of 17 β-estradiol in pharmaceutical preparation.

Intra-day				Inter-day	
Pharmaceutical preparation	Added	Found ± SD[a]	% Recovery% RSD[b]	Found ± SD[a]	% Recovery % RSD[b]
Spectrophotometry					
Estrofem (2 μg mL^{-1})	4	4.11 ± 0.049	102.8 (1.19)	4.16 ± 0.093	104.0 (2.24)
	7	7.04 ± 0.079	100.6 (1.12)	7.09 ± 0.155	101.3 (2.19)
	10	10.17 ± 0.078	101.7 (0.77)	10.22 ± 0.129	102.2 (1.26)
HPLC					
Estrofem (1000 ng mL^{-1})	500	497 ± 11.829	99.4 (2.38)	506 ± 16.546	101.2 (3.27)
	3000	3048 ± 77.419	101.6 (2.54)	3078 ± 98.188	102.4 (3.19)
	5000	4970 ± 93.933	99.4 (1.89)	5040 ± 129.528	100.8 (2.57)

[a] SD: Standard deviation of six replicate determinations.
[b] RSD: Relative standard deviation.

Table 5 Application of proposed methods for determination of 17 β-estradiol in Estrofem tablet.

Method	*n*	17 β-estradiol (mg)	Found ± SD (mg)	% RSD	% Recovery	*t*- test
Spectrophotometry	12	2	2.032 ± 0.0384	1.89	101.6	$t_t = 2.179$
HPLC	12	2	2.004 ± 0.0603	3.01	100.2	$t_c = 0.588$

n: Number of determination, SD: Standard deviation, RSD: Relative standard deviation, t_c: calculated *t*-value, t_t: tabulated *t*-value.
Ho hypothesis: no statistically significant difference exists between two methods, $t_t > t_c$; Ho hypothesis is accepted ($P > 0.05$).

sample analyses, the tailing factor was $\leqslant 1.12$, efficiency $\geqslant 2650$ and % RSD $\leqslant 1.83\%$. The % RSD of peak area and retention time for 17 β-estradiol are within 2% indicating the suitability of the system.

4. Discussion

For the UV method, various solvent systems investigated were high pure water, methanol and acetonitrile. The final decision for using methanol as the solvent was based on sensitivity, ease of preparation, suitability for drug content determination and stability studies, time and cost. The λ_{max} of 17 β-estradiol in methanol was found to be 281 nm and the corresponding UV spectra are shown in Fig. 2.

In case of HPLC, mobile phases investigated were 20–70% methanol in water and 20–70% acetonitrile in water. Mobile phase of methanol–water (70:30, v/v) and flow rate selection were based on peak parameters (height, asymmetry, and tailing), baseline drift, run time and ease of preparation of the mobile phase. A typical chromatogram for 17 β-estradiol using C_{18} column with mobile phase composition of methanol–water at 1.0 mL min^{-1} flow rate is shown in Fig. 3.

A study of some potential interfering substances in the UV and HPLC determination of 17 β-estradiol was performed by selecting them as the excipients often used in pharmaceutical preparation formulation. Samples containing a fixed amount of the 17 β-estradiol (1.0 μg mL^{-1}) and variable concentrations of excipients (talc, gelatin, lactose, corn starch, magnesium stearate, methyl hydroxypropyl cellulose, indigotin and titanium dioxide) were measured. All the results obtained by using the methods described above were compared with each other and no significant difference was observed between the amount of drug found as theoretical values for *t* at $P = 0.05$ level for commercial formulation.

United States Pharmacopoeia (The United States, 2000) has recommended liquid chromatography (HPLC) method for the analysis of related substances in pure 17 β-estradiol and assay of 17 β-estradiol in pharmaceutical dosage form (tablet). The method recommended the use of a mobile phase of 2,2,4-trimethylpentane-n-butyl chloride-methanol (45:4:1, *v*/*v*) at a flow rate of 2 mL min^{-1}, using UV detection (280 nm) on a stainless steel column (5 μm, 4.6 × 25 cm i.d.).

The proposed methods are very effective for the assay of 17 β-estradiol in pharmaceutical preparation. The validity of the proposed methods was presented by recovery studies using the standard addition method. For this purpose, a known amount of reference drug was spiked to formulated tablets and the nominal value of drug was estimated by the proposed method. Each level was repeated six times. No internal standard was used as no extraction step was involved in the estimation of 17 β-estradiol in pharmaceutical preparation. Also, the accuracy of the results established no need for internal standard for the suggested HPLC method.

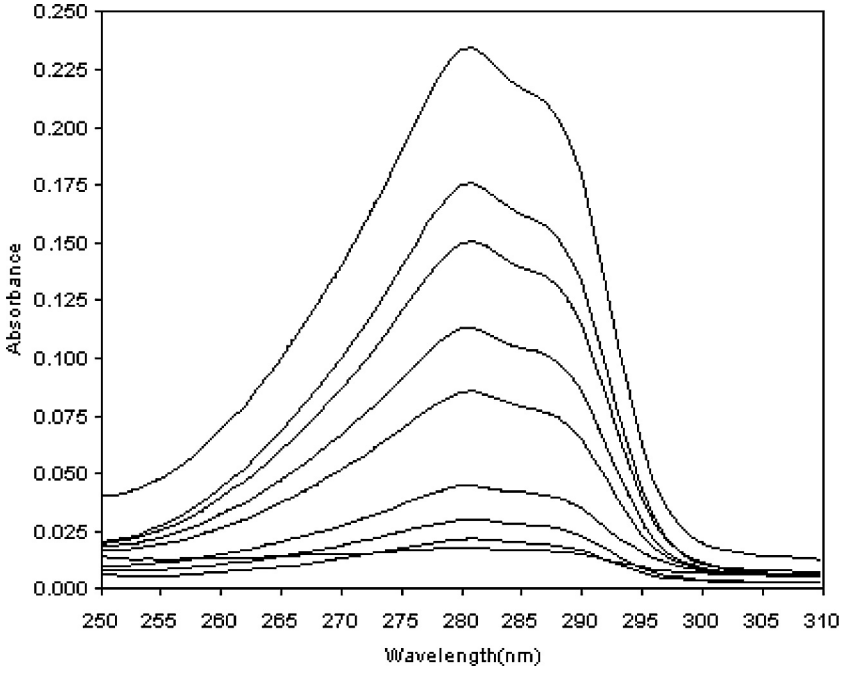

Figure 2 UV spectrum of 17 β-estradiol (0.5, 1, 1.5, 2, 4, 6, 8, 10 and 12 μg mL^{-1}).

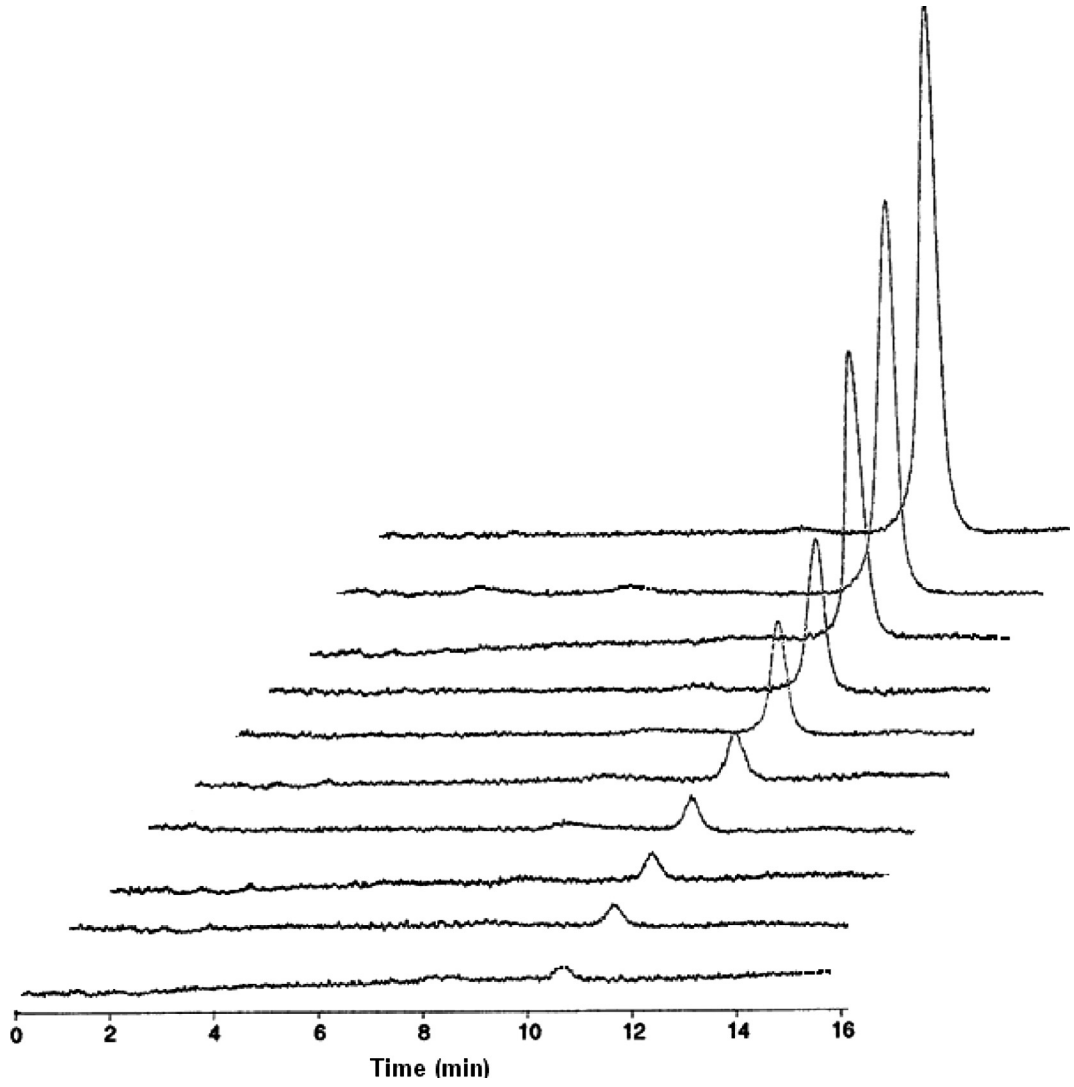

Figure 3 HPLC chromatogram of 17 β-estradiol (50, 125, 250, 500, 750, 1500, 2000, 4000, 5000 and 6000 ng mL^{-1}).

In comparison with earlier reported and official method for the estimation of 17 β-estradiol in pharmaceutical formulation the proposed HPLC method gave a lower LOD and LOQ values (Havlikova et al., 2006; The United States, 2000). Also, the HPLC method is found to be superior to earlier reported methods, as the mobile phase is simple to prepare and economical. On the other hand, the mobile phase is methanol consisting of water instead of buffered systems that are used in previously reported HPLC method (Yamada et al., 2002). The retention time of 17 β-estradiol was quite shorter than that that of earlier reported methods (Zacharia et al., 2004; Fotsis and Adlercreutz, 1987). No extraction procedure is involved and there is no need to use internal standard. Also, the medium for dissolving 17 β-estradiol is the same at HPLC and UV analysis. The sample recoveries in a formulation were in good agreement with their respective label claims (Table 5).

5. Conclusion

In the present report, simple, rapid, sensitive, reliable, specific, accurate and precise UV and HPLC methods for the determination of 17 β-estradiol in pharmaceutical preparation were developed and validated. The proposed methods can be used effectively, without separation and interference, for routine analysis of 17 β-estradiol in pure form and its formulation and can also be used for dissolution or similar studies. On the other hand, the UV method is also suitable for the analysis of sample during accelerated stability studies, routine analysis of formulations and raw materials.

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