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Separation of Δ6- and Δ9,11-estradiol: analytical method development, validation and practical application

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For estradiol (E2) the separation of the degradation products Δ6- and Δ9,11-E2 is especially challenging due to their structural similarity. There is no method described in the literature yet which adequately addresses this problem. The present study describes a HPLC method for the separation and quantitation of E2 and its degradation products 6α-hydroxy-E2, 6β-hydroxy-E2, 6-keto-E2, Δ9,11-E2, β-equilenol and Δ6-E2. The method employs a Kinetex™ PFP analytical column, using methanol and deionized water as mobile phases. Different UV- and fluorescence detection modes were used for maximal sensitivity and specificity. The applicability and capability of the method was demonstrated for Vagifem® tablets. Finally, the method was validated with respect to selectivity, sensitivity, linearity, precision and accuracy.

1. Introduction

Validated and therefore stability indicating test methods are a prerequisite for achieving mass balance for a given chemical entity or a pharmaceutical formulation. In 2005 Smela (Smela 2005) characterized a stability indicating method as "specific so that the quantity of the active ingredient, degradation products and other components of interest may be accurately measured without interference in the material being tested". To meet this requirement a test method has to be capable – among others – to quantify individual degradation products (Bakshi and Singh 2002). Hence, the chromatographic separation of all degradation products is of particular importance for chromatographic procedures (Bakshi and Singh 2002). Any co-elution of degradation products as well as inadequate removal from the chromatographic column or elution without chromatographic interactions will most likely cause inaccurate test results, for example in case of degradation products with different chromophores and therefore different photometric responses in the chosen detection mode (Bakshi and Singh 2002). If not properly considered a reliable mass balance cannot be achieved.

A variety of methods has been published addressing the assay of estradiol (E2) and its separation from other hormones. For example, E2 was determined in water and other environmental samples (Laganà et al. 2001; Ingrand et al. 2003; Ternes et al. 2002), in human hair (Choi et al. 2000), in human and rat body fluids (Lamparczyk et al. 1994; Zacharia et al. 2004; Yamada et al. 2002), in chicken tissue (Wang et al. 2011) as well as in pharmaceutical products (Wilson 2009; Yilmaz and Kadioglu 2010; Gatti et al. 1998).

Only few methods are described in the literature which concentrate on the separation of E2 from its degradation products (Nováková et al. 2004; Havlíková et al. 2006). Additionally, there is no concluding paper on the identity of all so far known E2 degradation products. Several analytical studies were performed following the photodegradation (Mazellier 2008), ozonation (Bila et al. 2007), biological transformation (Skotnicka-Pitak et al. 2008) or radiation-induced decomposition (Kimura et al. 2004) of E2. However, a comprehensive list of E2 degradation products occurring during storage of pharmaceutical products is still missing. Nygaard et al. (2004) postulated 6-keto-E2, Δ6-E2, 6α-hydroxy-E2, 6β-hydroxy-E2, 16-keto-E2, 6-keto-E2, β-equilenol and estron as E2 degradation products (as well as β-equilenol, estron 17α-E2 and 4-methyl-E2 as impurities) and developed a HPLC method to separate these compounds. Δ9,11-E2 was regarded only as a by-product from the synthesis (with an elution time close to Δ6-E2) and was left unconsidered during method development (Nygaard et al. 2004). In contrast to our experience Δ9,11-E2 also emerges during storage and thus has to be taken into account for method development. Up to now there is no published method which allows for a full separation of Δ6- and Δ9,11-E2.

We were able to achieve this goal by using a Kinetex™ PFP analytical column for separation. Additionally, E2 and all relevant degradation products were quantified using UV- and fluorescence detection. The applicability and suitability of the HPLC method – with respect to the above mentioned criteria for a stability indicating method – was confirmed using the marketed product Vagifem® (25 μg E2 in 80 mg tablet matrix) as model formulation.

For this drug product a special sample preparation procedure had to be developed due to the character of the tablet matrix and the very low drug substance to excipient ratio. Nygaard et al. (2004) already published an intricate sample preparation procedure with recoveries which left room for improvement for some of the included compounds. Consequently, this procedure had to be improved and simplified. Finally, the method was validated according to pharmaceutical standards.

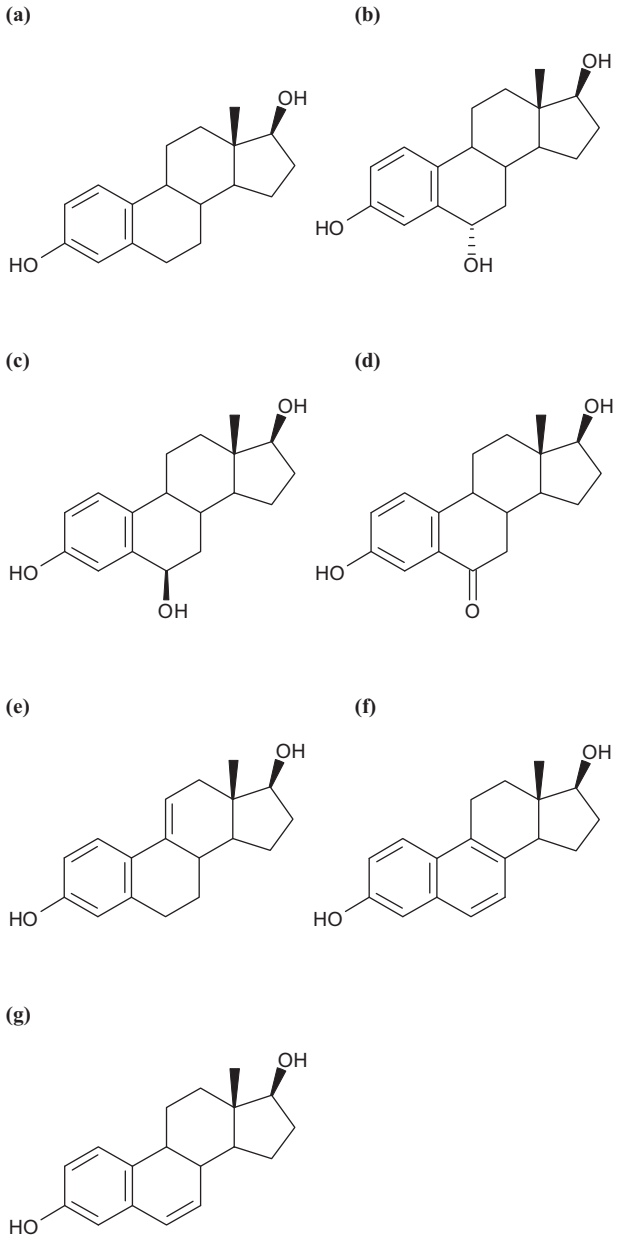

Fig. 1: Structures of the steroids included into the study. [a]: E2; [b]: 6α-hydroxy-E2; [c]: 6β-hydroxy-E2; [d]: 6-keto-E2; [e]: Δ9,11-E2; [f]: β-equilenol; [g]: Δ6-E2

2. Investigations, results and discussion

The structures of the steroids included into the study are presented in Fig. 1.

2.1. Selectivity

The selectivity of a method defines their capability to resolve different compounds in a sample as pre-requisite for unequivocal identification (Kromidas 1999). For chromatographic methods a resolution of two peaks greater than 1.4 is required for baseline separation (Rücker et al. 2001). In accordance with this definition a HPLC method was developed which separates E2 and all relevant degradation products – with special focus on the separation of Δ6- und Δ9,11-E2. The actual resolution of the individual compounds was verified on the basis of chromatograms recorded at 220 nm (Fig. 2). At this wavelength all compounds of interest are detectable. The resolution was calculated using Eq. (1) (Pharmacopoeia Europea 2011a):

$$\text{Resolution} = \frac{1.18\,(\text{retention time peak 2} - \text{retention time peak 1})}{\text{peak width at half height 1} + \text{peak width at half height 2}} \quad (1)$$

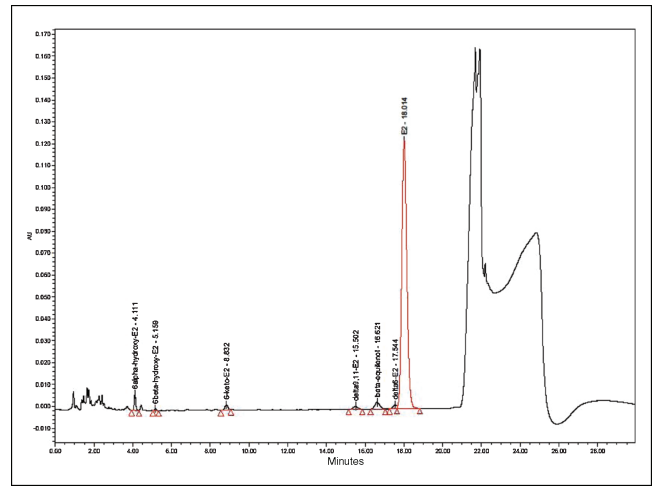

Fig. 2: Sample solution spiked with degradation products containing 100 µg/ml E2 and 0.5 µg/ml of 6α-hydroxy-E2 (containing 6β-hydroxy-E2), 6-keto-E2, Δ9,11-E2, β-equilenol and Δ6-E2, respectively. The chromatogram was detected at 220 nm using UV detection mode

Table 1: E2 and its degradation products: Resolution of adjacent peaks

Compounds	Resolution
6α-Hydroxy-E2 / 6β-hydroxy-E2	6.5
6β-Hydroxy-E2 / 6-keto-E2	15.5
6-Keto-E2 / Δ9,11-E2	20.2
Δ9,11-E2 / β-Equilenol	2.6
β-Equilenol / Δ6-E2	2.4
Δ6-E2 / E2	1.4

The calculated results are given in Table 1. 6α-Hydroxy-E2, 6β-hydroxy-E2, 6-keto-E2, Δ9,11-E2, β-equilenol, Δ6-E2 and E2 are well separated with the current analytical method. Even Δ6- and Δ9,11-E2 are for the first time completely separated (see Fig. 3) with a resolution of 6.2.

The resolutions between the peaks are even better than shown in Fig. 2 (220 nm recording) when evaluating the individual peaks on the basis of their specific fluorescence signals (wavelengths as listed in Table 2). Therefore, in practice all adjacent peaks are well resolved.

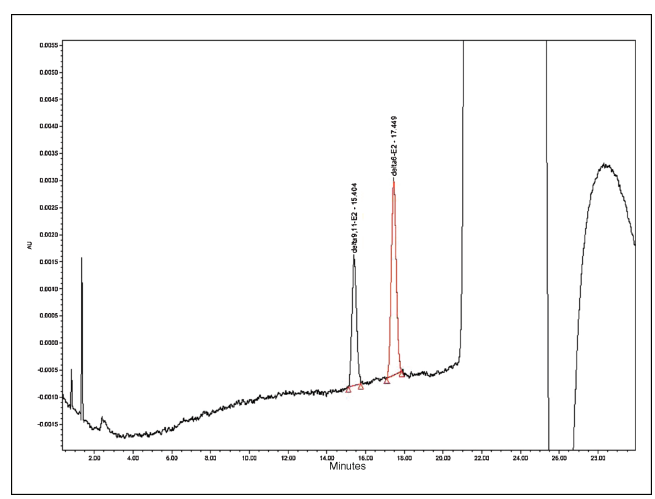

Fig. 3: Standard solution of Δ6- and Δ9,11-E2 containing 1.0 µg/ml of each compound. The chromatogram was detected at 220 nm using UV detection mode

Table 2: Detection modes and detection wavelengths for E2 and E2 degradation products

Compound	UV detection wavelength (nm)	FLD detection extinction wavelength (nm)	FLD detection emission wavelength (nm)
6α-Hydroxy-E2	(220)*	280	315
6β-Hydroxy-E2			
E2			
6-Keto-E2	220	–	–
Δ9,11-E2	(220)*	260	360
β-Equilenol	(220)*	220	360
Δ6-E2	(220)*	280	370

* Compound is visible at 220 nm but quantitation is performed using fluorescence detection mode

Table 3: LOD and LOQ of E2 degradation products

Compound	LOD (μg/ml)	LOQ (μg/ml)
6α-Hydroxy-E2	0.01	0.04
6β-Hydroxy-E2	0.02	0.06
6-Keto-E2	0.02	0.07
Δ9,11-E2	0.01	0.07
β-Equilenol	0.01	0.04
Δ6-E2	0.08	0.25

Nevertheless, the present method can be used with minor instrumental effort (with UV detection at 220 nm, without fluorescence detection) if the required LOD and LOQ may be higher than determined in this study with Vagifem® (e.g. for E2-formulations with a higher drug load). This time saving opportunity would be reasonable for routine application and formulations with negligible placebo interference.

2.2. Limit of detection (LOD), limit of quantitation (LOQ)

LOD and LOQ were calculated (as described in the Pharmacopoeia Europea 2011) with signal to noise ratios of 3 and 10, respectively. The determination initially was done with solutions of the analytes in matrix-free solvent (deionized water/methanol 3 + 2 (v/v)) and confirmed for the E2 degradation products by spiking freshly prepared tablet sample solutions with the calculated concentrations of the degradation products. The LOD and LOQ values are summarized in Table 3.

LOQ ranged from 0.04 μg/ml for 6α-hydroxy-E2 and β-equilenol to 0.25 μg/ml for Δ6-E2. These values correspond to concentrations of 0.04 % and 0.25 % relative to the drug substance. The ICH guideline Q3B requires a reporting threshold for degradation products of 0.1 % (ICH 2006) and therefore a limit of quantitation of less than that. Nevertheless, taking into account the very low drug substance concentration in the formulation and the low drug to excipient ratio (as already mentioned by Nygaard et al. (2004)) the LOQ of 0.25 % for Δ6-E2 relative to the drug substance is fully acceptable. Additionally, further degradation of Δ6-E2 leads to Δ9,11-E2, which is recorded with a LOQ of 0.07 %. Consequently, E2 and all degradation products can be detected and adequately quantified at very low concentration levels.

2.3. Linearity

Three independent standard solutions of the analytes were prepared in matrix-free solvent (deionized water/methanol 3 + 2 (v/v)). E2 solutions with concentrations of 50%, 100% and 150% as well as 0.1%, 0.25%, 0.5%, 1.0% and 2.0% of the E2 concentration in an authentic sample solution, which equates to 100 μg/ml, were prepared by dilution. Freshly prepared sample solutions were spiked with corresponding amounts of degradation products in order to confirm the linearity in presence of tablet matrix. The concentration levels were 0.1% (0.1 μg/ml), 0.25% (0.25 μg/ml), 0.5% (0.5 μg/ml), 1.0% (1.0 μg/ml) and 2.0% (2.0 μg/ml), except for Δ6-E2. In consideration of the higher LOQ for Δ6-E2 different concentration levels were chosen for this compound (0.25% (0.25 μg/ml), 0.5 % (0.5 μg/ml), 1.0% (1.0 μg/ml), 2.0% (2.0 μg/ml) and 5.0% (5.0 μg/ml)). Linearity was shown for all analytes within the tested intervals and with correlation coefficients of 0.998 or higher (Table 4). With regard to the ICH guideline for validation procedures (ICH 2005) and Limberg (2012) also the plots for residuals from the linear regression analyses were used for the evaluation. Two examples for these plots are presented in Fig. 4 and Fig. 5.

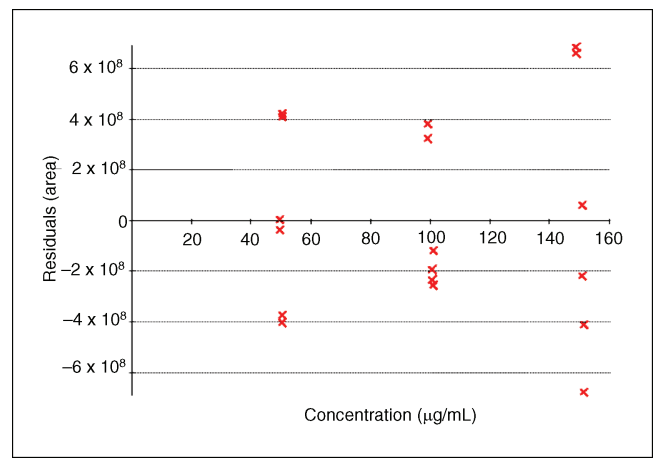

Fig. 4: Plot for residuals from linear regression analysis for E2 in the 50–150 % range

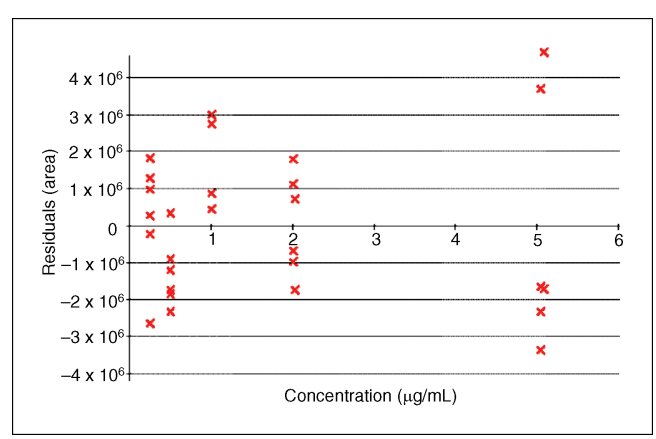

Fig. 5: Plot for residuals from linear regression analysis for Δ6-E2 in the 0.25–5.0 % range

Table 4: Linearity of E2 and E2 degradation products

Compound	y = a x + b	R^2	Confidence interval of y-axis intercept
6α-Hydroxy-E2	y = 189563263 x + 1280479	0.9999	± 668990
6β-Hydroxy-E2	y = 218370385 x + 1042847	0.9998	± 846125
6-Keto-E2	y = 43437 x + 167	0.9995	± 488
Δ9,11-E2	y = 559298602 x - 19925570	0.9977	± 13270403
β-Equilenol	y = 1484599227 x - 23888960	0.9997	± 13526612
Δ6-E2	y = 67403520 x + 68704	0.9998	± 1019863
E2 (0.1%−2.0%)	y = 262310690 x - 775526	0.9979	± 6178280
E2 (50%−150%)	y = 251922443 x + 782417484	0.9987	± 88779,669

y: peak area; x: concentration [μg/ml], a: slope; b: intercept; R^2: correlation coefficient

Table 5: Method precision for E2 and E2 degradation products

Compound	Coefficient of variation (%)	Coefficient of variation (%)	
		Inter-day precision (3 days)	Inter-day precision (7 days)
6α-Hydroxy-E2	4.5	5.2	5.3
6β-Hydroxy-E2	5.1	4.9	5.4
6-Keto-E2	6.9	5.9	5.8
Δ9,11-E2	6.6	6.3	6.5
β-Equilenol	7.5	7.8	7.2
Δ6-E2	6.5	6.5	7.1
E2	0.7	0.6	0.7

2.4. Precision

Method precision for E2 was determined by preparing six individual sample solutions. For estimation of the method precision for degradation products the initial sample solution was spiked with these compounds prior to sample preparation and extraction with a concentration of 0.5% (0.5 μg/ml) relative to the nominal E2 concentration in the sample solution (100 μg/ml). For each component the means peak areas and the corresponding coefficients of variation were calculated (Table 5).

The coefficient of variation for the drug substance E2 was less than 1% (0.7%). All coefficients of variation for the degradation products ranged between 4.5% (for 6α-hydroxy-E2) and 7.8% (for β-equilenol).

According to the Horwitz equation (Horwitz 1982; Boyer et al. 1985) coefficients of variation up to 8% are regarded as fully acceptable for testing analytes in the concentration range of E2 in the sample solution. For lower analyte concentrations (in the 1ppm range or below) coefficients of variation higher than 16% are considered adequate. All coefficients of variation for the method precision determined during this study were found to be within these generally accepted limits. The present method can therefore be regarded as suitable to determine E2, 6α-hydroxy-E2, 6β-hydroxy-E2, 6-keto-E2, Δ9,11-E2, β-equilenol and Δ6-E2 with sufficient precision.

2.5. Accuracy

Accuracy was determined by recovery. Three independent standard solutions of E2 and the degradation products were prepared. Each solution was diluted three times. Sample solutions were spiked with equivalent concentrations. The E2 recovery was determined on the 50%, 100% and 150% concentration level. For 6α-hydroxy-E2, 6β-hydroxy-E2, 6-keto-E2, Δ9,11-E2 and β-equilenol the recovery was determined on the 0.1%, 0.5% and 1.0 % level (relative to the nominal E2 concentration in the sample solution), while Δ6-E2 was tested at 0.25%, 0.5% and 1.0%. Additionally, six sample solutions were prepared to determine the assay of E2 (and potential degradation products) in the formulation.

The recovery was determined separately for each concentration level (and finally averaged) using Eq. (2):

$$\text{Recovery}[\%] = \frac{\text{Average peak area spiked sample solutions} - \text{Average peak area sample solutions (assay)}}{\text{Average peak area standard solutions}} * 100\% \quad (2)$$

For all calculated results see Table 6.

The recovery for E2 was 101.8%. Even though tested on much lower concentration levels the recoveries for 6α-hydroxy-E2, 6β-hydroxy-E2 and 6-keto-E2 were in accordance with the recommendation of 95.0% to 105.0% for concentrations < 1.0% relative to the drug substance (Kromidas 1999) with rates of 104.2%, 104.6% and 104.1%, respectively. For β-equilenol the

Table 6: Accuracy: Presented are the recoveries of E2 and E2 degradation products in Vagifem® at three different concentration levels. The average value represents the total recovery for each compound

Compound	Recovery[1] (%)	Recovery[2] (%)	Recovery[3] (%)	**Total recovery (%)**
6α-Hydroxy-E2	111.7	101.5	99.4	**104.2**
6β-Hydroxy-E2	101.8	98.2	113.8	**104.6**
6-Keto-E2	109.4	103.7	99.1	**104.1**
Δ9,11-E2	115.9	124.6	122.3	**121.0**
β-Equilenol	103.4	112.0	108.6	**108.0**
Δ6-E2	93.5	89.5	87.1	**90.0**
E2	103.0	101.3	101.0	**101.8**

[1] E2: 50% level; degradation products (except Δ6-E2): 0.1 % level; Δ6-E2: 0.25 % level (see LOQ)
[2] E2: 100% level; degradation products: 0.5 % level
[3] E2: 150% level; degradation products: 1.0% level

recovery was found to be 108.0%. Nevertheless, taking into account the method precision for β-equilenol (7.5%) the deviation from 100.0% is very close to the method precision and a recovery of 108.0% is therefore well acceptable. The recovery of Δ6-E2 was determined with 90.0%. According to Kromidas (1999) a recovery rate smaller than 100% is fully acceptable as long as the deviation of the actual recovery from 100% is not more than the fourfold coefficient of variation of the method precision. Thus, with a coefficient of variation of 6.5% the recovery rate for Δ6-E2 (90.0%) is considered well acceptable. Additionally, Δ9,11-E2 recovery was found to be within the fourfold coefficient of variation (6.6%) with 121.0%. Moreover, this offers a significant improvement compared to Nygaard et al. (2004), who did not consider Δ9,11-E2 at all and described single recovery values for compounds up to 191.7%. Nevertheless, for routine application the introduction of a correction factor for the recovery should be taken into consideration.

2.6. Conclusion

A HPLC method for the complete separation and quantitation of E2 and all relevant degradation products in a pharmaceutical formulation with a very low E2 content was successfully developed and validated (without robustness). For the first time the separation of Δ6- and Δ9,11-E2 was achieved. This method is therefore appropriate for mass balance investigations on E2-containing pharmaceutical products. The applicability and capability of the method even for low-dose formulations was demonstrated for the marketed drug product Vagifem® as example. A specific sample preparation procedure was developed and incorporated in the final method validation. Furthermore, this HPLC method is suitable for the analysis of other E2-containing pharmaceutical products. The method is a compromise between optimal resolution, analysis time, efforts for sample preparation and - last not least - robustness. A further improvement in terms of speed and resolution can possibly be achieved by using sub-2 μm capillary columns in combination with high back pressure (>1000 bar) pump systems. However, this work was primarily focussed on the development of a method for the complete separation of the relevant degradation products of estradiol on the basis of established robust standard HPLC equipment.

3. Experimental

3.1. Chemicals and drug products

E2 hemihydrate reference standard (96.7%), 6α-hydroxy-E2 reference standard (84.1% containing 6β-hydroxy-E2 8.8%), 6-keto-E2 reference standard (99.4%) and β-equilenol reference standard (98.5%) were supplied by Bayer AG (Bergkamen, Germany). Δ9,11-E2 reference standard (96.8%) was purchased from LGC GmbH (Luckenwalde, Germany). Δ6-E2 reference standard (98.7%) was acquired from Dalton (Toronto, Canada).
Water was purified with a Milli-Q® Advantage A10 system (Millipore, Schwalbach, Germany). Methanol was purchased in HPLC gradient grade from Merck (Darmstadt, Germany). Acetone was purchased in HPLC grade from Sigma Aldrich (Seelze, Germany).
Vagifem® is a trademark of Novo Nordisk Pharma GmbH (Mainz, Germany) and a registered drug product in Germany (purchased via pharmacy). Its active pharmaceutical ingredient is E2 hemihydrate; the excipients are hypromellose, lactose monohydrate, maize starch, magnesium stearate and macrogol 6000.

3.2. HPLC analysis

HPLC analysis was performed using an Agilent 1100 series HPLC system consisting of pumps, degasser, autosampler, column heater, a photodiode array detector and a fluorescence detector (Agilent technologies, Waldbronn, Germany).
In order to obtain a stability indicating method, the separation of all E2 degradation products had to be achieved (Fig. 1). Especially the separation of Δ6- and Δ9,11-E2 appeared to be difficult as it has not been described in the literature yet.
In this study different column types (like ProntoSIL 120 C18 AQ, Zorbax Eclipse C18 XDB, Ascentis® Express C18, Hypercarb™, ZirChrom®-Carb) and a variety of elution gradients were tested to achieve this separation. In addition, existing chromatographic methods for the separation of E2 and E2 impurities were tested (Pharmacopoeia Europea 2011b; Nygaard et al. 2004).
Finally, a Kinetex™ 2.6 μm PFP column (150 × 4.6 mm) from Phenomenex (Torrance, USA) was selected as stationary phase. The mobile phase was composed of deionized water and methanol with a volume ratio of 55:45 at the start of the elution gradient (Table 7). Until 13.0 min the eluent ratio was changed to 51.4:48.6. Within the same period the flow rate was changed from initially 1.0 ml/min to 0.7 ml/min until 22.5 min, when it was switched back to 1.0 ml/min. The eluent composition was 50.0% deionized water and 50.0% methanol at min 18.0 and 100.0% methanol at min 18.5. At min 23.0 the eluent ratio was set to starting conditions for column re-equilibration. The column temperature was 60 °C throughout analysis. Each sample solution was injected twice and peak areas were averaged. In order to achieve very low limits of detection (LOD) and quantitation (LOQ) as well as to minimize influences of matrix compounds in the chromatograms, the detection modes were optimized separately for each analyte by evaluating UV- and fluorescence spectra. Individual detection wavelengths were identified for the compounds of interest (Table 2). For data collection and analysis the Empower 2 software (Waters, Milford, USA) was applied.

Table 7: Gradient of the mobile phase and the flow rate. A: deionized water, B: methanol

Time (min)	Flow (ml/min)	A (%)	B (%)
0	1.0	55.0	45.0
13.0	0.7	51.4	48.6
18.0	0.7	50.0	50.0
18.5	0.7	0	100.0
22.5	1.0	0	100.0
23.0	1.0	55.0	45.0

3.3. Extraction procedure

For practical application of the HPLC method a marketed tablet formulation with a very low concentration of E2 was chosen (Vagifem®). As described by Nygaard et al. (2004) the extraction of E2 and its degradation products from the tablet matrix is demanding because of a drug to excipient ratio of 1:3200. Consequently, 25 μg E2 (and even lower concentrations of E2 degradation products after storage) had to be completely extracted from tablets with 80 mg tablet weight. In addition, a hypromellose (hydroxypropylmethylcellulose, HPMC) is part of the formulation, which forms a gel-like structure whenever it gets in contact with water so that a quantitative extraction of the analytes of interest from the tablet matrix might become impossible. Therefore, a non-aqueous extraction method was applied. Moreover, due to the low drug to excipient ratio in the tablets a concentration step was implemented in order to increase the concentrations of the analytes in the final sample solution. Finally, the requirements for the extraction solvents were good solubility of E2 and its degradation products, low solubility for matrix compounds (especially HPMC), absence of water and the possibility of (fast) evaporation to concentrate the sample solution. Several solvents (like methanol, acetonitrile, toluene, chloroform, tetrahydrofuran, water and even mixtures of the solvents in different concentrations) were tested. Only acetone met all of the defined requirements and was chosen as extraction solvent. Nygaard et al. (2004) developed an extraction procedure based on 16 h magnetic stirring as well as several concentration and extraction steps. For our procedure an optimized and time-saving sample preparation was established.
15 tablets were pestled, weighed in a 25 ml volumetric flask and made up to volume with acetone. Extraction was performed by 30 min magnetic stirring. The dispersion was centrifuged for 10 min with approximately 2000–2500 g. 10.0 ml of the clear supernatant were withdrawn and evaporated to dryness under nitrogen flow. The dry residue was redissolved in 1.5 ml deionized water/methanol 3 + 2 (v/v) and represented the final sample solution with a nominal E2-concentration of 100 μg/ml.

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