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Development and validation of a reversed-phase liquid chromatographic method for analysis of degradation products of estradiol in Vagifem® tablets

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Abstract

A stability-indicating liquid chromatographic method for the determination of degradation products and impurities in Vagifem®, estradiol vaginal tablets has been developed and validated. Vagifem® is a low dose preparation containing only 25 μg 17β-estradiol in a tablet matrix of 80 mg (a drug to excipient ratio of 1:3200). This paper presents the rationale for the optimization of the sample preparation in order to minimize placebo interference as well as validation data for linearity, accuracy, precision, ruggedness, specificity and limits of detection and quantification. Data shows that the method is suitable for routine analysis of minute amounts of estradiol impurities.

Keywords: Degradation products; Estradiol; Pharmaceutical analysis; Placebo interference; Reverse phase chromatography; Sample preparation; Stability indicating

1. Introduction

Vagifem®, estradiol vaginal tablets, is a marketed product for the relief of post-menopausal atrophic vaginitis due to estrogen deficiency.

Vagifem® is a low dose preparation containing only 25 μg 17β-estradiol (=E2) in a tablet matrix of 80 mg and consequently the ratio of drug to excipient is very low (equal to 1:3200) compared to most other tablet products. The excipients included in the composition of the Vagifem® tablet are stated in Table 1. Hydroxyl propyl methyl cellulose, lactose and starch are the major components.

A stability indicating method for determination of chromatographic degradation products/impurities was originally developed and validated with satisfactory results. In this original sample procedure tablets were added to ethanol and extraction was performed by means of magnetic stirring. The suspension was centrifuged and the supernatant was evaporated to dryness. The residue was re-dissolved in ethanol and then centrifuged. The supernatant

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Table 1
Composition of Vagifem® and solubility characteristics

Compound	Function	Solubility characteristics [10,11]	
		Water	Ethanol
E2	Active ingredient	Almost insoluble	Freely soluble
Hydroxyl propyl methyl cellulose (HPMC)	Binder	Soluble (cold)	Practically insoluble
Lactose	Filler	Freely soluble	Practically insoluble
Starch	Filler	Practically insoluble	Practically insoluble
Magnesium stearate	Lubricant	Practically insoluble	Practically insoluble
Polyethylene glycol	Coating	Soluble	Soluble

was the sample solution (E2 concentration = 0.096 mg/ml).

Ethanol was chosen as extraction solvent, because E2 and E2 impurities are soluble in this solvent whereas the dominant excipients are practically insoluble in ethanol. In spite of the expected selectivity of ethanol, the chromatogram of placebo in Fig. 1 shows that complete removal of interference was not accomplished as the chromatogram contained several peaks. The reason was probably that even though only small amounts of excipients were dissolved, the heavy concentration of the sample (160 times) during the extraction procedure meant that these minute amounts resulted in significant placebo peaks in the chromatogram.

However, as placebo interference in general is undesired and in this specific case made the interpretation of the impurity profile of E2 in the tablets more difficult it was decided to optimize the sample preparation in order to minimize interference.

A number of especially HPLC methods describing the analytical testing of steroids have been published. These papers focus on the determination of the assay [1–3] and of the impurity profile of various steroids, several including structural characterization of individual impurities [4–8]. In [9], it is demonstrated that TLC is capable to monitor the impurity and degradation profile of tablets containing E2.

However, the aim of this paper is to attract attention on the optimization of the sample preparation procedure of a low dose tablet formulation and the simultaneous development and validation of a stability indicating HPLC method which is capable of determining degradation products/impurities of synthesis of E2. The low drug to excipient ratio has represented some unique challenges for the development of a suitable method for routine analysis especially concerning the concentration of the sample solution in order to achieve a satisfactory limit of quantification for the impurities as well as (and at the same time) minimizing the placebo interference.

Data supporting linearity, accuracy, precision, ruggedness, specificity and limits of detection and quantification are presented.

2. Experimental

2.1. Equipment

For development and validation the following configuration was used. The HPLC instrument was from Waters (Milford, MA, US) and consisted of Waters 717 autosampler, Waters 510 pumps or Waters 515 pumps, Waters 2487 detector and Waters Millenium software system. Cooling of the autosampler was used. A photo diode array (PDA) detector, Waters 996, was used to collect spectral data of E2 and E2 impurities.

The analytical column was a symmetry C18, 5 μm, 250 mm × 4.6 mm (i.d.) from Waters.

2.2. Materials

E2, USP Reference Standard, Lot No. K was used throughout the study as reference material.

The following impurities were obtained from Steraloid Inc (Wilton, NH, US): 6α-hydroxy-E2, 6β-hydroxy-E2, 6-keto-E2, 16-keto-E2, 6-keto-estrone, β-equilenol, 6-dehydro-E2 and 4-methyl-E2. The impurities α-E2 and estrone were obtained from Diosynth (Oss, NL). The structure of E2 and the impurities is shown in Table 2.

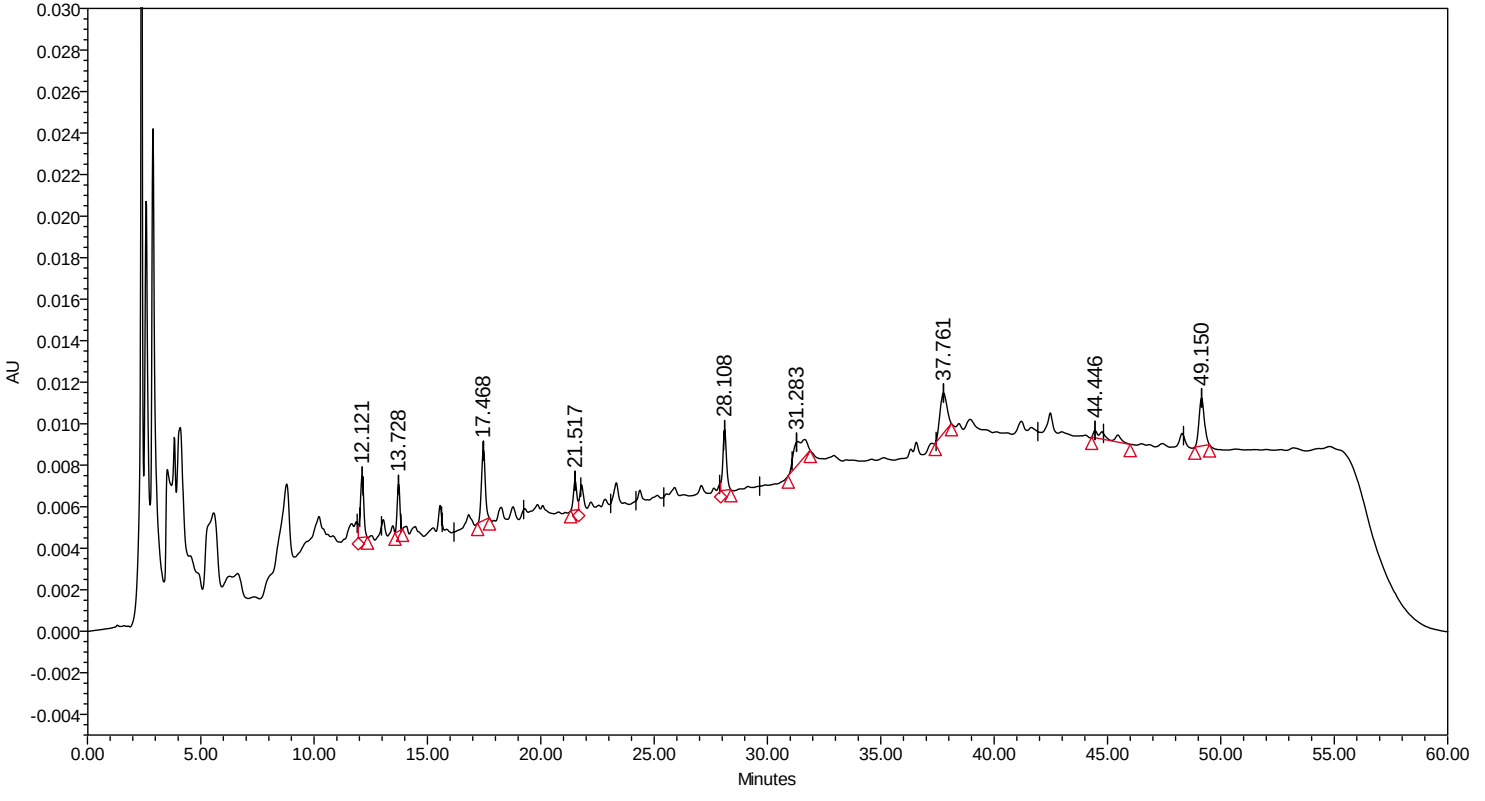


Fig. 1. Chromatogram of a placebo solution before optimization of sample preparation showing multiple placebo interference.

Table 2
Structure of E2 and E2 impurities

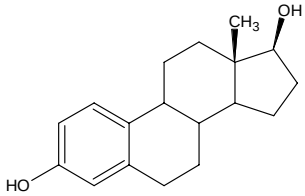
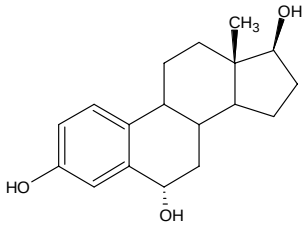
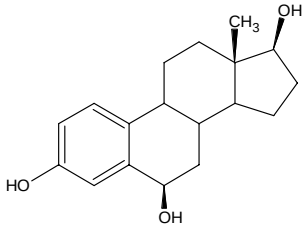
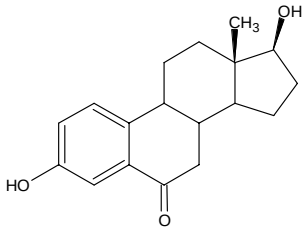
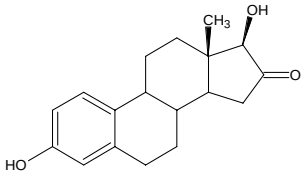
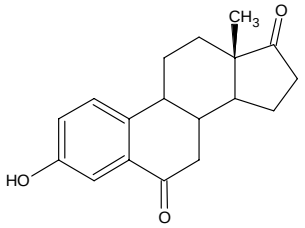
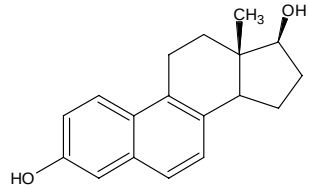
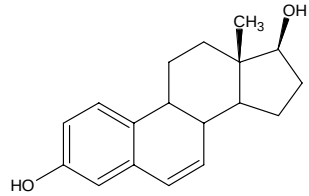
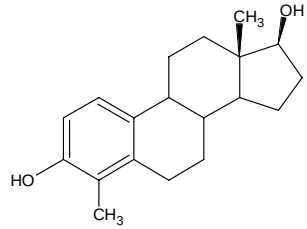
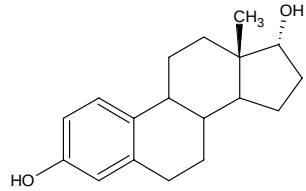
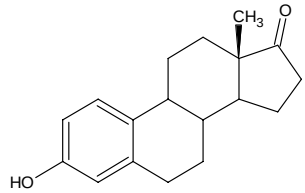
Name of compound	Structure
E2	
6α-Hydroxy-E2	
6β-Hydroxy-E2	
6-Keto-E2	
16-Keto-E2	
6-Keto-estrone	

Table 2 (*Continued*)

Name of compound	Structure
β-Equilenol	
6-Dehydro-E2	
4-Methyl-E2	
α-E2	
Estrone	

Acetonitrile, acetone, chloroform, ethanol, hexane, methyl ethyl ketone, methylene chloride and toluene were HPLC or analytical grade obtained from Rathburn or Merck. Milli-Q water (Millipore) was used.

The sample material (for both method development and validation) consisted of stability tablet batches as well as samples containing a synthetic mixture of 12 placebo tablets, E2 (corresponding to the amount of E2 in Vagifem®) and the mentioned E2 related substances.

The impurities represent a broad range of polarity in which the hydroxy compounds are the most polar impurities, 4-methyl-E2 being the least. 6-Keto-E2 and 6-dehydro-E2 are dominating degradation products of Vagifem®, 6α-hydroxy-E2 and 6β-hydroxy-E2 represents less abundant degradation products, whereas 16-keto-E2, 6-keto-estrone, β-equilenol and estrone are potential degradation products. The last two compounds as well as α-E2 and 4-methyl-E2 are also typical impurities of synthesis.

2.3. Chromatographic conditions

Mobile phase A was filtered and degassed Milli-Q water. Mobile phase B was filtered and degassed 80% (v/v) acetonitrile. E2 and E2 related substances was eluted by a linear gradient from 20% B to 85% B at 35 min. Hold for 14 min, return in 1.0 min to initial conditions and equilibrate for 10 min before next injection. Flow rate was 1.0 ml/min. Injection volume was 25 μl for standards and samples. The detection wavelength was 220 nm (PDA: scan from 190 to 400 nm).

2.4. Method development

2.4.1. Original sample procedure

Twelve tablets were added to 50 ml of the extraction solvent of ethanol and extraction was performed by means of magnetic stirring for 16 h. The suspension was centrifuged and 8.00 ml of the supernatant was evaporated to dryness. The residue was re-dissolved in 500 μl of ethanol and then centrifuged and used as sample solution.

2.4.2. Optimization of sample procedure

The following extraction procedures were investigated:

1. Solid–liquid extraction
   - Substitution of ethanol in the solid–liquid extraction of 12 tablets by different organic solvents.
2. Addition of a liquid–liquid extraction step
   - The solid–liquid extraction of 12 tablets in 50.00 ml of ethanol was maintained in order to obtain adequate recovery of E2 and E2 impurities and to have a solvent which was easy to concentrate by evaporation.
   - After centrifugation an aliquot of 10.00 ml of the ethanol sample solution was evaporated to dryness using nitrogen.
   - An additional liquid–liquid extraction step on the residue from the evaporation of the aliquot of 10.00 ml of ethanol was introduced by partitioning the sample between two immiscible phases, an aqueous and an organic phase.
   - The analyte was recovered by evaporation of the organic phase. The residue was redissolved in 450 μl ethanol and used as sample solution.

3. Results and discussion

3.1. Solid–liquid extraction

Instead of introducing an additional step of extraction a simpler approach was to optimize the first solid–liquid extraction of the 12 tablets in 50.00 ml of ethanol. Attempts were done in which ethanol was replaced by chloroform, acetone, toluene, methyl ethyl ketone or mixtures of water and the mentioned solvents. In all cases the placebo interference was significantly greater compared to the two-step procedure presented below. Taking the complex tablet matrix, as well as the rather huge requirement for concentration of the sample during sample treatment into consideration, it was expected that a two-step extraction procedure representing two different extraction principles and application of solvents with a broad range of polarity was more effective.

3.2. Liquid–liquid extraction

The rationale for optimization of the sample preparation using liquid–liquid extraction was based on the

fact that most of the excipients are soluble in water, whereas E2 (and E2 related substances as impurities of synthesis and degradation products) are practically insoluble in this solvent indicating that the partitioning of the sample between an aqueous and an organic phase might improve the selectivity of the extraction procedure.

Liquid–liquid extraction was investigated in the following way. The residue from the evaporation of 10.00 ml of ethanol sample solution was extracted using a mixture consisting of 1.00 or 2.00 ml of water and typical 5.00 ml of one of the following organic solvents, all immiscible with water: methylene chloride, chloroform, toluene and hexane (ranked after decreasing polarity). The first screening showed that the placebo interference was significantly reduced using all four solvents due to the transfer of the water soluble excipients to the aqueous phase during the step of liquid–liquid extraction. Toluene was selected for further experiments as the most significant reduction in placebo interference was achieved by this solvent and because of the much less toxicity compared to the other solvents.

Liquid–liquid extraction on a sample consisting of placebo, E2 and 10 E2 impurities using a mixture of 1.00 ml of water and 5.00 ml of toluene gave a recovery for 6α-hydroxy-E2 and 6β-hydroxy-E2 of approximately 5% and 60–100% for E2 and the other E2 impurities, respectively. The very low recovery of the two most polar impurities, 6α-hydroxy-E2 and 6β-hydroxy-E2, indicated that these compounds preferred the aqueous phase instead of the non-polar toluene phase. This was confirmed by analyzing the aqueous phase. By replacing toluene with the more polar solvent methylene chloride the recovery of 6α-hydroxy-E2 and 6β-hydroxy-E2 was increased to 70–75% (the recovery of E2 and the other E2 impurities was >90%). Even though the use of methylene chloride is undesirable from a toxicological point of view the experiment indicated that an increase of the polarity of the organic phase lead to an increase of the recovery for the most polar impurities.

The increase of polarity was instead achieved by adding acetone to the organic phase. This solvent has a polarity close to methylene chloride but are much more toxicological acceptable. Recovery experiments showed that a mixture of 1.00 ml of water + 2.00 ml of acetone + 5.00 ml of toluene gave satisfactory results for all impurities (Table 3), as well as significant reduction in placebo interference. This mixture was chosen as the final solvent for liquid–liquid extraction. Figs. 2 and 3 show typical chromatograms for a placebo and sample solution. A few peaks still remain in the placebo chromatogram. These are due to chromatographic and UV active impurities originating from excipients soluble as well as insoluble in ethanol. However, the reduced placebo interference compared to the original sample procedure (Fig. 1) is evident.

It should be mentioned that acetone is not immiscible with water, i.e. the basic principle for liquid–liquid extraction by separating analytes from interference by partitioning the sample between two immiscible phases is no longer fully obtained. However, examination of the above-mentioned mixture still showed two

Table 3
Effect of Acetone on recovery of individual impurities

Extraction solvent	20 °C (W/A/T: 1.0/2.0/5.0)	20 °C (W/A/T: 1.0/1.5/5.5)	20 °C (W/A/T: 1.0/2.5/4.5)	30 °C (W/A/T: 1.0/2.0/5.0)
6α-Hydroxy-E2	93.4	78.8	78.1	83.4
6β-Hydroxy-E2	97.0	78.4	86.1	89.2
6-Keto-E2	102.3	95.4	96.1	110.8
16-Keto-E2	98.7	93.0	84.0	76.0
6-Keto-estrone	110.2	98.8	104.0	97.6
β-Equilenol	103.0	93.0	94.7	95.4
6-Dehydro-E2	103.9	93.0	94.9	102.3
α-E2	104.6	94.1	100.7	95.9
Estrone	105.5	94.5	97.8	94.7
4-Methyl-E2	99.6	90.6	95.4	94.6

W/A/T: water/acetone/toluene.

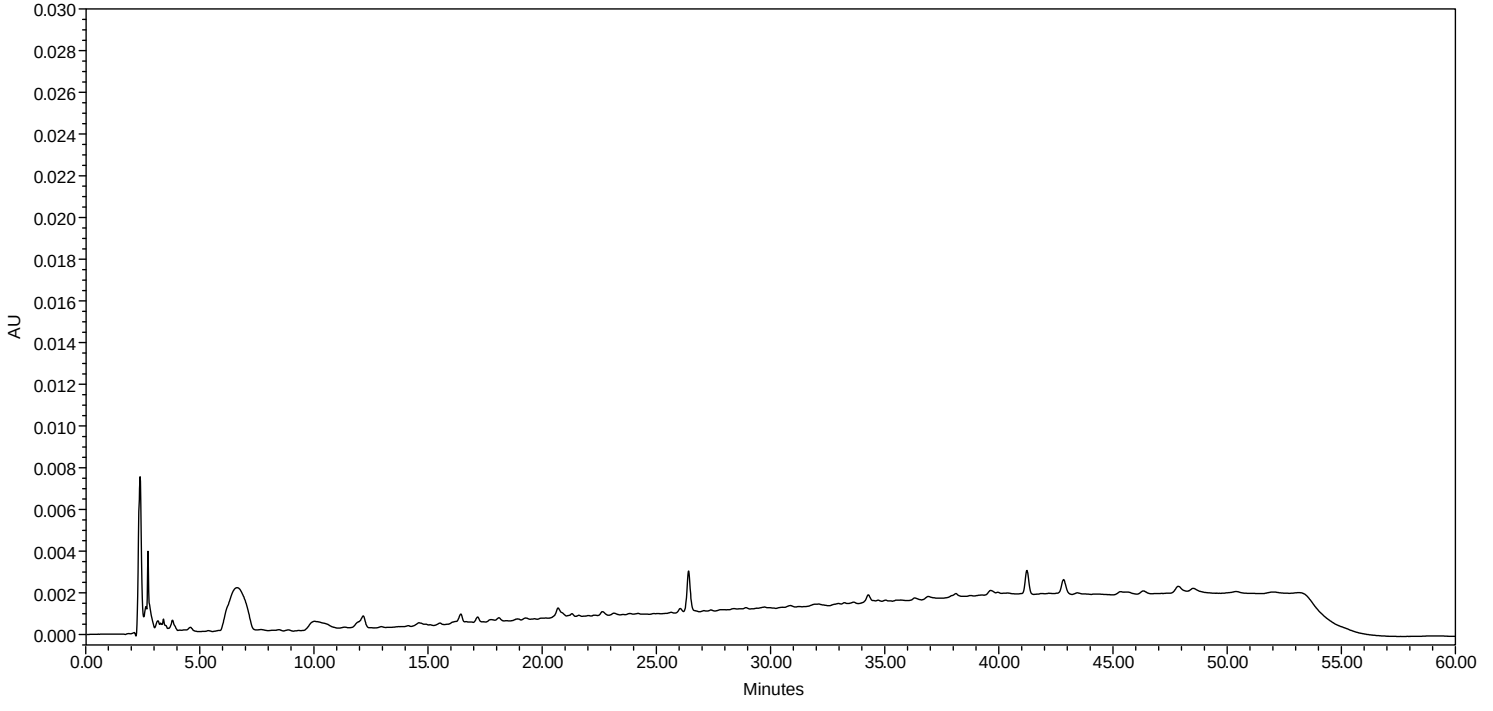


Fig. 2. Chromatogram of a placebo solution of the optimized method showing reduced interference.

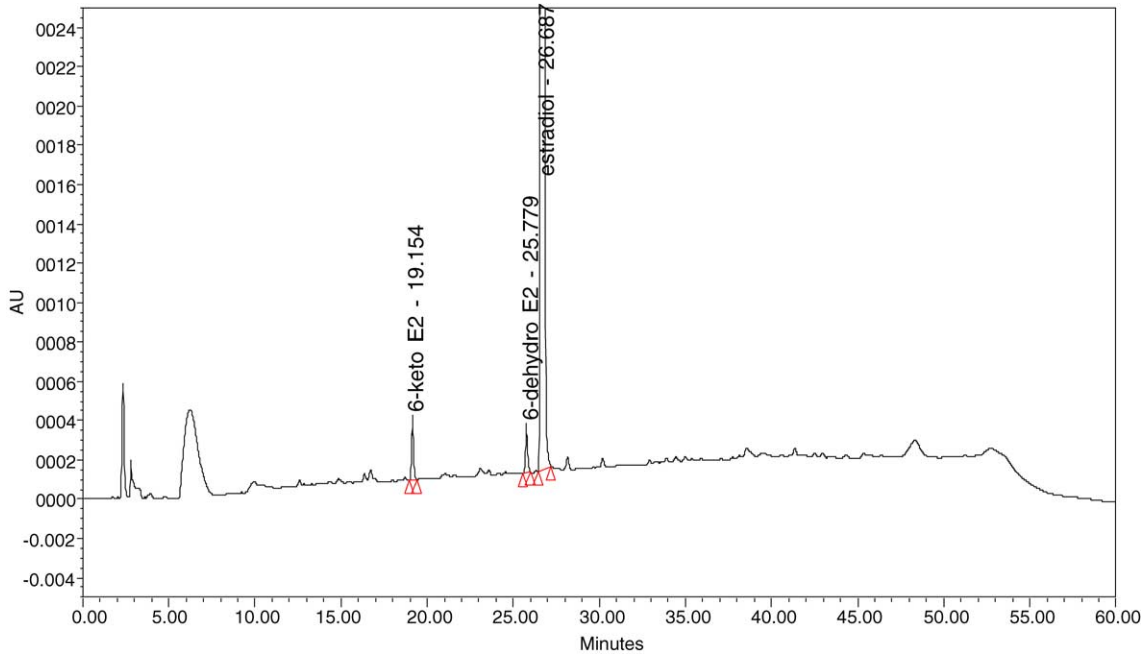


Fig. 3. Chromatogram of a stability sample solution.

distinct phases and only small changes in the volume of the aqueous phase and the organic phase, respectively.

The effect of variation of the volume of acetone and toluene was investigated by analyzing the 10 potential impurities added to a placebo batch. As can be seen from Table 3 small changes in the extraction solvent do not affect the final results.

The same table also shows that extraction at 30 °C means no significant difference compared to the previous results obtained at room temperature.

3.3. Specificity

The specificity of the method regarding placebo interference has already been discussed. Separation of E2 from impurities of synthesis and potential degradation products was demonstrated by injections of an E2 solution spiked with the following impurities: 6α-hydroxy-E2, 6β-hydroxy-E2, 6-keto-E2, 16-keto-E2, 6-keto-estrone, β-equilenol, 6-dehydro-E2, 4-methyl-E2, α-E2 and estrone. As can been seen in Fig. 4, all the relevant impurities were well separated from the E2 peak as well as from each other. Another well-known impurity of synthesis, 9(11)-dehydro-E2, elutes close to 6-dehydro-E2. Like the other impurities of synthesis, 9(11)-dehydro-E2 is controlled by the analysis of the E2 raw material.

Even though a significant decrease in placebo interference was obtained it was not possible to achieve a complete removal of placebo interference; therefore, a run of a placebo batch is included in the testing procedure in order to make it possible to discriminate between peaks originating from impurities and from excipients. Some of the placebo peaks co-elute or elute closely to some of the impurities (e.g. α-E2 and estrone). This may result in overestimating the content of impurities of E2, especially when the content of the individual impurities is low, i.e. close to the limit of quantitation. However, as discussed later satisfactory results for accuracy and precision were obtained during the validation of the method. Further, a placebo peak can be observed co-eluting with E2 (Fig. 2), but the area of this placebo contributes with less of 0.6% of total area in the E2 peak, therefore, correction is not needed.

The homogeneity of the peak of E2 was investigated by analysis of a tablet batch by means of a PDA

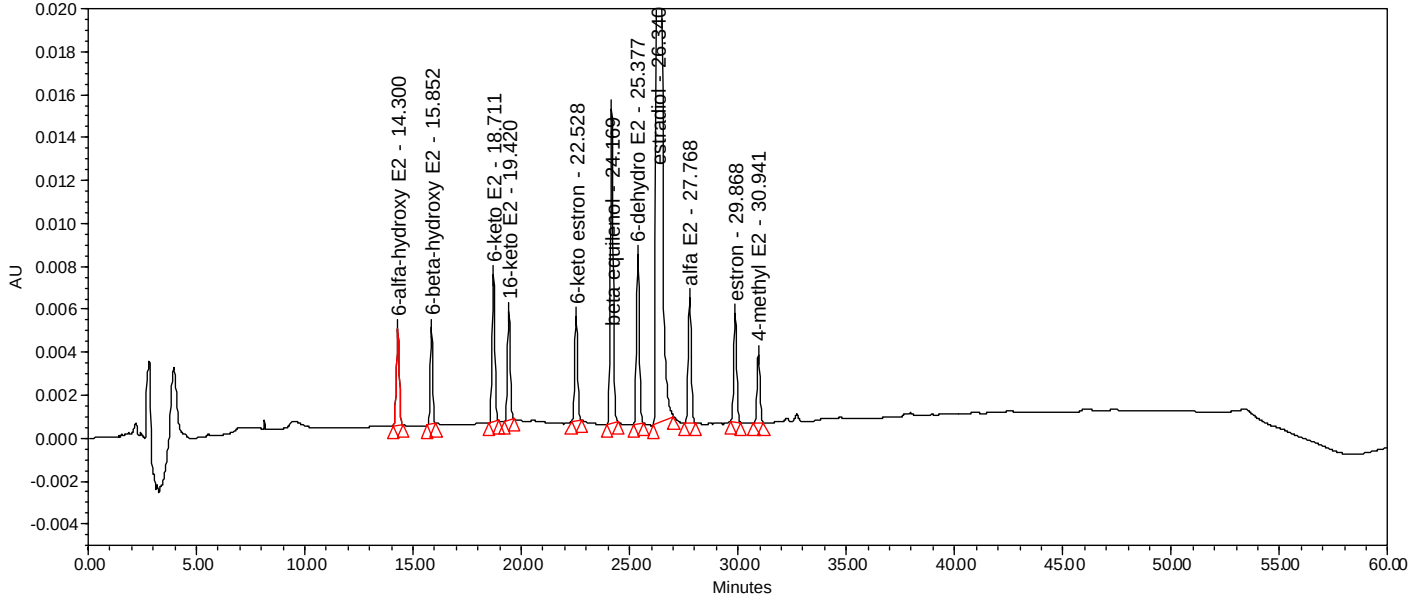


Fig. 4. Placebo solution spiked with estradiol and impurities. The solution contained 98 μg/ml E2, 1.05 μg/ml 6α-hydroxy-E2, 1.05 μg/ml 6β-hydroxy-E2, 0.525 μg/ml 6-keto-E2, 1.05 μg/ml 16-keto-E2, 0,525 μg/ml 6-keto-estrone, 0,525 μg/ml β-equilenol, 0,525 μg/ml 6-dehydro-E2, 1.05 μg/ml α-E2, 1.05 μg/ml estrone, 1.05 μg/ml 4-methyl-E2.

detector. UV spectra in the range of 190–400 NM was obtained during the elution of the E2 peak and subsequent calculations using Millenium software demonstrated peak homogeneity. It should be mentioned that this approach does not account for impurities having a UV-spectra identical to E2.

Identification of E2 peak in a sample solution was confirmed by spectral comparison with an E2 reference standard solution.

3.4. LOD and LOQ

In the presence of E2 and placebo the LOD and LOQ for 10 chromatographic impurities, defined as a signal to noise ratio of approximately 3 (S/N = 3) and 10 (S/N), respectively, have been determined by appropriate dilution of impurities in a solution containing 98 μg/ml estradiol and placebo.

In Table 4 are presented the results of six repetitive injections of the LOQ solution. The mean peak area and the R.S.D. of the injections were calculated. The relative content of impurities had been calculated (%) using E2 in equivalent amounts of a true sample concentration. Based on these results a LOQ of 0.3% is considered as a representative value. For 6-keto-E2, 6-dehydro-E2 and β-equilenol the chromophore has been significantly changed compared to E2 due to the presence of double bonds conjugated to the aromatic system. This means that the UV-response is higher for these impurities and consequently the LOQ values are lower. The high peak area of α-E2 is due to the fact that the E2 reference material in the LOQ solutions contained 0.2% of this impurity. The LOD was estimated to approximately 0.1% (results not shown).

The ICH Guideline [12] recommends 0.1% as a general reporting limit for individual impurities. However, 0.3% is considered as an acceptable value taken into consideration that Vagifem® 25 μg is a low dose preparation having a very low drug to excipient ratio. Compared to the original method the optimization means an improvement of LOQ (and LOD) by 40%.

3.5. Linearity

The linearity for E2 and the 10 potential impurities was determined. Linearity of E2 was determined at six levels 1, 2, 5, 10, 50 and 100%, where 100% corresponds to 98 μg/ml of E2. Three injections of each concentration level were performed.

USP recommends calculations of impurities based on a diluted reference solution, e.g. 2%. This is only a negotiable procedure, if sample extraction is complete. However, relating to the content of impurities to the E2 peak in the sample solution makes the quantitation less dependent of extraction efficiency. Since linearity has been shown, a calculation based on either the E2 peak area of a diluted reference solution or the E2 area of an actual sample is direct interchangeable.

Individual impurities were spiked to a placebo batch and linearity was determined at five concentrations corresponding to 1×LOQ, 2×LOQ, 4×LOQ, 8×LOQ

Table 4
Limit of quantification

Compound	LOQ		Peak area	
	μg/ml	Percentage of E2	Mean ($n = 6$)	R.S.D.
6α-Hydroxy-E2	0.303	0.31	10119	1.9
6β-Hydroxy-E2	0.288	0.29	8700	0.8
6-Keto-E2	0.110	0.11	10766	1.0
16-Keto-E2	0.289	0.29	11013	1.6
6-Keto-estrone	0.115	0.12	10750	1.2
β-Equilenol	0.057	0.06	10316	0.9
6-Dehydro-E2	0.082	0.08	12165	0.5
α-E2	0.274	0.28	19610	0.8
Estrone	0.283	0.29	12682	1.0
4-Methyl-E2	0.342	0.35	10131	1.1
Estradiol			3748646	0.5

Percentage of E2 calculated corresponding to an estradiol sample concentration of 98 μg/ml.

Table 5
Linearity of estradiol and estradiol related impurities

Compound	$Y = \alpha X + \beta$[a]	R^2
6α-Hydroxy-E2	$Y = 9161X - 214$	0.9879
6β-Hydroxy-E2	$Y = 7535X + 235$	0.9887
6-Keto-E2	$Y = 9358X - 672$	0.9948
16-Keto-E2	$Y = 8473X + 556$	0.9980
6-Keto-estrone	$Y = 9934X + 706$	0.9966
β-Equilenol	$Y = 8856X - 560$	0.9945
6-Dehydro-E2	$Y = 9572X - 495$	0.9954
α-E2	$Y = 9863X - 672$	0.9965
Estrone	$Y = 10345X + 11$	0.9965
4-Methyl-E2	$Y = 10221X - 1283$	0.9943
E2	$Y = 36730X - 271$	0.9999

Y: peak area; X: concentration; α: slope; β: intercept.
[a] Concentration in μg/ml.

Table 6
Accuracy and recovery of impurities

Compound	Recovery in % (conc.[a])	Recovery in % (conc.[a])	Recovery in % (conc.[a])
6α-Hydroxy-estradiol	98.0 ± 2.3 (0.30)	98.6 ± 9.4 (1.19)	83.5 ± 4.1 (2.38)
6β-Hydroxy-estradiol	103.7 ± 1.4 (0.28)	98.7 ± 9.7 (1.13)	82.5 ± 3.8 (2.26)
6-Keto-estradiol	118.7 ± 3.6 (0.11)	114.4 ± 9.4 (0.43)	100.8 ± 4.2 (0.86)
16-Keto-estradiol	103.3 ± 3.6 (0.28)	102.8 ± 7.0 (1.13)	91.9 ± 3.9 (2.26)
6-Keto-estrone	124.9 ± 3.0 (0.11)	114.7 ± 9.3 (0.45)	102.2 ± 3.2 (0.90)
β-Equilenol	122.9 ± 3.0 (0.06)	120.5 ± 8.9 (0.22)	105.1 ± 3.6 (0.45)
6-Dehydro-estradiol	141.2 ± 4.0 (0.08)	124.5 ± 9.2 (0.32)	105.8 ± 3.2 (0.65)
α-Estradiol	191.7 ± 2.5 (0.27)	138.1 ± 9.2 (1.07)	112.5 ± 3.3 (2.15)
Estrone	125.2 ± 2.5 (0.28)	119.3 ± 9.3 (1.11)	104.0 ± 3.4 (2.22)
4-Methyl-estradiol	107.1 ± 3.3 (0.34)	111.3 ± 9.0 (1.34)	99.7 ± 3.6 (2.67)

[a] Concentration in μg/ml.

and 10 × LOQ. Three injections of each concentration level were performed. All linearity parameters are given in Table 5. Satisfactory linearity is shown for all impurities in the tested interval.

3.6. Accuracy

The accuracy of the method with respect to E2 and the 10 possible impurities was determined. A solution containing the impurities and the active substance was added to 12 placebo tablets after which sample extraction proceeded. The placebo sample preparations were spiked in triplicate at three different levels corresponding to a content of impurities of approximately 1 × LOQ, 4 × LOQ and 8 × LOQ. The actual concentrations (μg/ml) and the recovery of the individual components are presented in Table 6. For calculation of the recovery three standard solutions covering the three levels of spiking were injected. It is seen that recovery is satisfactory and except for the α-E2 at the 1 × LOQ level in the range of 80–140%. The higher results for α-E2 is due to the content of low levels of α-E2 in the E2 reference solution as already mentioned.

Table 7
Precision of stability samples

		Area (%)	Total R.S.D. (%)	Between R.S.D. (%)	Intra R.S.D. (%)
Batch 1	6-Keto-E2	1.08	6.0	5.5	2.5
	6-Dehydro-E2	0.58	11.1	7.6	8.1
	%Sum	1.69	8.7	8.3	2.8
Batch 2	6-Keto-E2	1.51	3.6	3.0	2.0
	6-Dehydro-E2	0.80	8.3	5.1	6.5
	%Sum	2.36	6.5	6.2	2.0
Batch 3	6-Keto-E2	0.63	7.0	5.4	4.4
	6-Dehydro-E2	0.47	7.0	2.5	6.6
	%Sum	1.38	20.7	9.1	18.6
Batch 4	6-Keto-E2	0.63	3.9	3.8	1.1
	6-Dehydro-E2	0.46	5.6	3.4	4.5
	%Sum	1.35	25.4	16.6	19.2
Batch 5	6-Keto-E2	0.67	4.3	3.9	1.7
	6-Dehydro-E2	0.55	7.3	3.4	6.5
	%Sum	1.48	21.1	8.1	19.5

Calculation of precision is based on analysis of variance, but given as R.S.D. (%). Total = $(\mathrm{var}_{\mathrm{Between}} + \mathrm{var}_{\mathrm{Intra}})^{1/2}$. Between: between days. Intra: between duplicates.

3.7. Precision

The precision study included six tablet batches representing a various degree of degradation which were analyzed at five individual days using two different HPLC systems. All sample preparations were made in duplicate—although the final method uses one sample preparation. Repeatability, reproducibility and intermediary precision of the method were investigated as a combined study. Statistic calculations were carried out by means of analysis of variance (i.e. ANOVA) and corresponding R.S.D. values were calculated.

The five batches from stability studies contained primarily 6-keto-E2 and 6-dehydro-E2. In Table 7, a summary of the results is presented, it is seen that the R.S.D. for the individual impurities varies between 3 and 11%, which is satisfactory. However, for "sum of impurities" the obtained R.S.D.-values were in some cases higher. This is due to peaks close to LOQ, which may be just above LOQ in some HPLC runs and in others just below.

Taken the low concentration level into consideration good precision within and between runs has been demonstrated and 11% is used as a conservative estimate of the analytical precision.

References

[1] J.A.P. Gluck, E. Shek, J. Chromatogr. Sci. 18 (1980) 631–636.
[2] G.M. Sundaresan, T.J. Goehl, V.K. Prasad, J. Pharm. Sci. 70 (1981) 702–704.
[3] R.B. Miller, C. Chen, Chromatographia 40 (1995) 204–206.
[4] S. Görög, B. Herényi, J. Chromatogr. 400 (1987) 177–186.
[5] S. Görög, A. Laukó, B. Herényi, J. Pharm. Biomed. Anal. 6 (1988) 697–705.
[6] S. Görög, J. Brlik, A. Csehi, Z. Halmos, B. Herényi, P. Horváth, F. Dravetz, D. Bor, Anal. Methods Instrum. 2 (1995) 154–158.
[7] S. Görög, M. Babják, G. Balogh, J. Brlik, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó, K. Varga, J. Pharm. Biomed. Anal. 18 (1998) 511–525.
[8] I.V. Ekhato, T. Hurley, M. Lovdahl, T.J. Revitte, L. Guo, Y. Huang, S. Clipper, C. Colson, Steroids 67 (2002) 165–174.
[9] K. Ferenczy-Fodor, V. Végh, Identification and Determination of Impurities in Drugs, in: S. Görög (Ed.), Elsevier, Amsterdam, 2000, pp. 146–182.
[10] A.H. Kibbe (Ed.), Handbook of Pharmaceutical Excipients, Third ed., Pharmaceutical Press, London and Chicago, 2000.
[11] The Merck Index, 13th ed., Merck & Co., Inc., Rahway, NJ, USA.
[12] ICH Harmonised Tripartite Guideline, Impurities in New Drug Products, ICH secretariate, Geneva, Switzerland.