ISSN 0946-1965

DOI 10.5414/CP201589
e-pub: December 2, 2011

Bioavailability and pharmacodynamics of two 10-mg estradiol valerate depot formulations following IM single dose administration in healthy postmenopausal volunteers

Barbara S. Schug, Frank Donath and Henning H. Blume

SocraTec R&D, Oberursel, Germany

Key words
estradiol valerate – estradiol – estrone – bioavailability – bioequivalence – pharmacokinetics – pharmacodynamics – postmenopausal – hormone replacement therapy

Abstract. Objective: To establish the bioequivalence (BE) between two i.m. estradiol valerate (E2V) depot formulations, i.e., Estradiol-Depot 10 mg® (test) and Progynon Depot-10® (reference). To compare the effect of both treatments on the vaginal maturation index and on the increase of the endometrial thickness after administration of both formulations. Methods: A total of 24 postmenopausal females aged 54.7 ± 5.35 year (BMI 25.84 ± 1.98 kg/m^2) completed this BE assessment. The investigation was planned and designed as a single center, open-label, single dose, cross-over study including 2 periods with 2 treatments and 2 sequences. Baseline levels were obtained for all subjects. Single doses of 10-mg E2V of each product were administered and pharmacokinetics and pharmacodynamics were assessed over 2 weeks with a washout period of 4 weeks. A gas chromatographic-mass spectrometric method with negative chemical ionization and selected ion monitoring was applied, after validation, for the determination of estradiol (E2), estrone (E1) and internal standard estradiol-D4 derivatives. The cytology of the vaginal smear (parabasal, intermediate and superficial cells from lateral wall opposite tip of cervix) was assessed by investigation of ~ 200 cells. The vaginal maturation index (VMI) was calculated by the equation: VMI (%) = (superficial cells × 1) (%) + (intermediate cells × 0.5) (%). Endometrial thickness was measured by transvaginal ultrasonic scans and recorded in mm. Results: The geometric means (Gmeans) of the measured values of C_{max} and AUC_{0-t} for E2 were 543.5 pg/ml and 84,734 pg × h/ml for test and 505.7 pg/ml and 82,660 pg × h/ml for reference, whereas those for E1 were 219.0 pg/ml and 38,950 pg × h/ml for test and 204.9 pg/ml and 37,159 pg × h/ml for reference, respectively. The point estimates (PEs) of the Test/Reference (T/R) mean ratios of the variables C_{max} and AUC_{0-t} for E2 (measured values) were 107.3% and 102.5%, respectively. The PEs of the T/R mean ratios of the variables C_{max} and AUC_{0-t} for E1 (measured values) were 106.9% and 105.0%, respectively. Median endometrial thickness increased in Period I from baseline levels of ~ 3 mm (Day –2) to ~ 7 mm (Day 21) after administration of both products without returning completely to baseline prior to the next administration. In Period II, median values of 7 mm were also reached (Day 21) after administration of both products. Median vaginal maturation indices increased in Period I from baseline levels of ranging from 45 – 60% (Day –2) to 86 – 94.5% (Day 21). In Period II maturation indices of ≥ 90% were calculated as baselines (Day –2) and these levels remained constant until the end of the assessment (Day 21) independently from the products. After 21 days of treatment, test and reference presented practically no differences in terms of their effects on endometrial thickness and vaginal maturation index. Conclusions: The 95% CIs for the T/R mean ratios of AUC_{0-t} and C_{max} for E2 and E1 fell within the acceptance limits of 80 – 125% and therefore bioequivalence could be demonstrated for both formulations. The changes in endometrial thickness and the vaginal maturation index indicated that the pharmacodynamic effect is pronounced already after the first administration and that the effect continued notably for longer time compared to the presence of E2 and E1 in plasma. A 4-week washout phase was insufficient to avoid residual pharmacological effects after the administration of both preparations.

Received
May 9, 2011;
accepted
August 18, 2011

Correspondence to
Barbara S. Schug, PhD
SocraTec R&D, Im
Setzling 35, 61440
Oberursel, Germany
Barbara.Schug@
socratec-pharma.de

Introduction

Hormone replacement therapy in postmenopausal women intends to improve symptoms caused by an estrogen deficiency [1]. Therefore, the close association between pharmacokinetics, indicated by the time course of serum or plasma concentrations of sex steroids after administration, and pharmacodynamics, indicated by the metabolic

and biological changes caused by the treatment, shows the fundamental importance of pharmacological knowledge for the use of hormone therapy [1].

Estradiol, more exactly 17β-estradiol (E2), is the most potent and important naturally occurring, endogenous estrogen [1, 3]. Beyond its essential role in reproduction, it affects the whole organism and is involved in many metabolic processes. E2 is synthesized in the growing ovarian follicles and the corpus luteum, the placenta, adrenals and Leydig cells, but also in the liver, endometrium, brain, muscle and fat tissue [1]. During an ovulatory cycle, the serum concentrations of E2 vary from 30 pg/ml in the early follicular phase, to 150 – 350 pg/ml in the preovulatory phase and 100 – 210 pg/ml in the luteal phase. During pregnancy, the estradiol levels rise 100-fold, reaching concentrations of ~ 20,000 pg/ml at the end of the third trimester. On the contrary, in postmenopausal women, the estradiol levels are usually below 20 pg/ml [1].

For therapeutic purposes, E2 is commonly administered as different fatty acid esters. Some of them, e.g. estradiol valerate (E2V), have demonstrated to exhibit pharmacokinetic and pharmacodynamic profiles after oral administration similar to those of estradiol [2], primarily due to its rapid hydrolysation to estradiol already in the intestinal mucosa [4, 5]. The metabolic pathway of estradiol is completed by further metabolites such as estrone and estrone sulphate, which are the main products of its hepatic biotransformation [4]. In normal healthy women (27 ± 6 years), it has been reported that following a single oral dose of 8-mg E2, serum concentrations of the parent compound of 18 ± 18 pg/ml (mean ± SD) are observed after 48 h, whereas the E1 concentrations were 197 ± 217 pg/ml (mean ± SD) [6]. Depending on the indication and the intention of the treatment, oral administration of E2 may not be adequate for long-term therapy, either because non-sufficient plasma levels are reached or due to compliance problems. To overcome this obstacle, the use of the intramuscular route presents an alternative to the oral administration. The intramuscular administration (i.m.) of E2V commonly is done by means of the injection of an oily solution of the E2 ester. In this solution, the solvent is absorbed, and a primary depot of the crystalline estradiol ester is formed at the site of injection. Thereafter, a secondary depot of the same substance can be found in the fat tissue [1, 2]. The formation of such depots permits the slow release of the ester, whereas its hydrolysation and further metabolisation are completed at the liver and in other organs [2]. Former studies in 9 healthy volunteers following the intramuscular injection of 5 mg of E2V have shown that geometric mean values of maximum plasma levels of 667 pg/ml (95% CI = 450.60 – 983.29 pg/ml, CV = 36.1%) for E2 and 321.74 pg/ml (95% CI = 202.77 – 513.70 pg/ml, CV = 43.6%) for E1 are observed after 24 – 48 h, decreasing to pre-administration levels after 9 – 11 days [7]. Furthermore, studies in postmenopausal women with a similar dose of E2V, i.e. 4 mg, showed maximum plasma levels ranging from 310 to 570 pg/ml for E2 and of ~ 100 pg/ml for E1 after 3 – 5 days, which decreased below 50 pg/ml for E2 and E1 after 20 days. [8]. According to these data, it can be seen that the use of a depot formulation results in higher estradiol levels for prolonged time.

Estradiol, like any other estrogen, is a promoter of the tissue growth in the reproductive organs of a woman; therefore, the collective effect of E2 can be estimated through the evaluation of the superficial, intermediate and parabasal cells of the vaginal mucosa in a test denominated vaginal maturation index [9]. This test can be accompanied by the determination of the endometrial thickness, taking into account that the endometrium contains estrogen receptors responding to the circulating estrogens [10].

The primary objectives of this study were the characterization of the relative bioavailabilities of two different oily i.m. depot solutions containing 10 mg of estradiol valerate each, the assessment of the bioequivalence of both products, and the simultaneous comparative assessment of the pharmacodynamic effects of both formulations through gynecological surrogate parameters, i.e., the change in vaginal maturation index and the increase of thickness of the endometrium, as no information about the timely relation between plasma levels and effect on the aforementioned parameters has been determined so far. A gas chromatographic-mass spectrometric (GC-MS) method with negative chemical ionization (NCI) and selected ion

monitoring (SIM) was validated and applied for the determination of indicative ions of estradiol and estrone. The application of this method allowed avoiding the overestimation of concentrations commonly reported when immunoassay kits are applied [11].

Methods

A single-center, open-label, single-dose, randomized crossover study with baseline assessment, two periods and two treatment sequences was used to compare 1-ml oily solutions containing 10.0 mg E2V each. In both solutions, the vehicle was composed of benzyl benzoate and castor oil. The test product was obtained from Jenapharm, Jena, Germany, and the reference product was purchased from Schering, Berlin, Germany.

A maximum of 36 volunteers with an interim analysis after 24 volunteers were planned to undergo the same crossover treatments, as no valid information on intraindividual variability was available for a reliable sample size estimation. For baseline assessment, blood sampling was performed over 48 h prior to each administration. The evaluated products were administered in the morning as slow single i.m. injections into the gluteal muscle after a 12-h fasting period with blood sampling over 14 days following i.m. administration. A washout period of 4 weeks between the two treatments, i.e. test and reference product, was maintained. Gynecological examinations and endometrial scans were done before and during each treatment. The study was performed according to GCP-requirements and the latest revision of the Declaration of Helsinki.

Subjects

A total of 24 healthy postmenopausal female subjects, planned age 45 – 75 years and a body mass index (BMI) from ≥ 20 to ≤ 29 kg/m^2 was included. Ethic's vote was obtained by the Medical Board of the State of Thuringia, Germany, prior to any measures. Subjects underwent an informed consent procedure prior to enrolment in accordance with Good Clinical Practice and national law. Major selection criteria were the lack of any relevant deviations from normal findings for healthy subjects as well as the absence of any other concomitant disease in the clinical findings that could interfere with the safety (in particular any risk for thromboembolic disorders) or study objectives. Postmenopausal state was confirmed by plasma FSH ≥ 40 IU/l and plasma estradiol ≤ 20 pg/ml, and last spontaneous menstruation ≥ 2 years ago or bilateral ovariectomy of at least 3 months ago. Besides, a normal gynecological examination, a normal transvaginal scan with < 5 mm of endometrial thickness, and a cytological smear classified according to Papanicolau with a pap-grading not higher than II were required. Hysterectomized subjects and volunteers with medical history of alcohol or drug abuse were excluded. The use of drugs inducing or inhibiting liver enzymes, diuretics, anticoagulants, digoxin, and antibiotics was not permitted within 8 weeks before the first drug administration. The use of sex hormones was not permitted within 6 weeks (oral, transdermal, and vaginal preparations) or 2 months (i.m. depot preparations administered once per month) or 6 months (i.m. depot preparations administered once per 3 months) before the first study drug administration and until the end of the study. All subjects had to avoid regular excessive physical activity and were non-smokers or mild smokers (5 cigarettes/day or less).

Sample preparation

During both treatment periods, blood samples for E2 and E1 analysis were taken at ~ 48, 24, 12, and 0.5 h prior to each dose for baseline assessment as well as at 1, 3, 6, 10, 16, 24, 36, 48, 60, 72, and 96 h and at 5, 6, 8, 10, 12, and 14 days after each dosing. Samples were centrifuged within 0.5 h after withdrawal for 10 min at 2,000 × g at room temperature; the supernatant was transferred into polypropylene storage tubes (2 aliquots per sample) and stored frozen below –20 °C until analysis. For sample work-up frozen plasma samples were thawed in a water bath at 20 °C and homogenised using a vortex shaker for 10 s. Afterwards samples were spiked with 20 µl of a methanolic solution of α-naphthol (c = ~ 100 ng/µl) and swirled anew. Extraction started by addition of 6 ml

of a mixture of n-pentane : ethylacetate = 7 : 3 (v/v), followed by mixing in a rotary mixer and centrifugation at 4,000 rpm for 10 min. The upper organic layer was transferred into a 10 ml reaction vial (with screw cap) and evaporated under a stream of nitrogen in a 50 °C water bath to dryness. The dry residues underwent two derivatisation steps. The first one consisted of the treatment of the dry residue with 50 µl of a 1% solution of 2,3,4,5,6-pentafluorobenzyl-chloride and 50 µl of a 0.2% solution of triethylamine (both reagents dissolved in dichloromethane), briefly swirled and left at room temperature for 15 min. Afterwards reaction mixtures were dried under a stream of nitrogen in a 50 °C water bath. In the second derivatisation, the dried samples of the first derivatisation were treated with 100 µl of n-hexane, briefly swirled and spiked with 50 µl of trifluoroacetic anhydride. Reaction vials were immediately closed by screw caps, mixed again and placed into a drying cabinet for 45 min at 65 °C. Afterwards samples were cooled down for 5 min and evaporated to dryness (nitrogen, 50 °C). Dried residues were reconstituted in 40-µl toluene and transferred into 200-µl microvials. Samples of 2 µl were used for analysis.

Analytical method validation

The applied analytical procedure was based on a gas chromatographic-mass spectrometric (GC-MS) method with negative chemical ionisation (NCI) and selected ion monitoring (SIM) of indicative ions of estradiol, estrone and estradiol-D4 derivatives. Within this method, an estradiol-D4 derivative was used as internal standard (IS). The estradiol (E2) and estrone (E1) derivatives were estrone-pentafluorobenzoyl (E1-PFBz derivative) and estradiol-pentafluorobenzoyl-trifluoroacetic anhydride (E2-PFBz-TFA derivative). As IS estradiol-d4-PFBz-TFA-derivative was used. The method validation was performed as proposed by Shah et al. [12] and considering the recommendations provided by the US-FDA Guidance for Industry, Bioanalytical Methods Validation for Human Studies [13]. The fundamental parameters for this validation included accuracy, precision, specificity, sensitivity, reproducibility, and stability.

Sample analysis

For analysis, calibration curves for E2 and E1 were prepared in a range of 10.0 – 250.0 pg/ml. The lower limit of quantification (LLOQ) applied for both analytes, i.e., E2 and E1, was 10.0 pg/ml. E2 and E1 derivatives were evaluated by relating the integrated analyte signal to the corresponding integrated IS derivative signal. Calibrations of the analyte substances were done by linear regression functions after 1/x-weighting of analyte/internal standard peak area ratios vs. analyte concentration. All analyses were done by Pharmakin GmbH, Ulm, Germany and fulfilling the criteria defined by Shah et al. [10] by the US-FDA Guidance for Industry, Bioanalytical Methods Validation for Human Studies [13].

Pharmacokinetic analysis

All pharmacokinetic (PK) parameters of E2 and E1 were determined model-independently using WinNonLin, Version 2.1 (Pharsight Cooperation, Mountain View, CA, USA) at Quintiles ClinData, Bloemfontein, South Africa. The parameters were determined directly from the measured plasma concentrations. Moreover, net values of mean plasma concentrations, obtained by subtracting the means of each of three basal concentrations at –48, –24 and –12 h from the individual measured mean plasma concentrations at each sample point, were calculated and a second set of E2 and E1 net PK-parameters was established, i.e., PK parameters based on net values. In the case of negative values obtained as net values, they were set to zero for calculation of PK parameters. Net values at Time 0 were set to zero by definition. All LLOQ values in the absorption phase were used as half LLOQ. LLOQ values (or the basal value) in the terminal phase were used as half LLOQ for calculation of the mean concentration-time profiles and as missing values for the calculation of the PK variables.

Primary PK parameters were: AUC_{0-t} (area under the concentration-time curve of the analyte in plasma over the time interval from 0 to the last quantifiable analyte plasma concentration (LLOQ) after single dose administration) as calculated from the linear

trapezoidal rule and C_{max} (maximum concentration of the analyte in a concentration-time profile). Secondary PK endpoints were $AUC_{0-\infty}$ (area under the plasma concentration-time curve from time 0 extrapolated to infinity) for the net values only, t_{max} (time from dosing to C_{max}), $\%AUC_{tz-\infty}$ (percentage of $AUC_{0-\infty}$ that was extrapolated from the time of the last quantifiable data point to infinity) and $t_{1/2}$ (apparent terminal half-life).

Gynecologic and safety examinations

Gynecological examinations, endometrial scans and cytological determinations were done by trained and experienced specialists in Erfurt, Germany, at pre-study, on Days –2, 8, 14, and 21 in each treatment period and post-study only in case of an endometrial thickness > 5 mm. Cytological smears were performed during prestudy investigation and classified according to Papanicolau class system (Pap I – V). Special attention was put on the detection of clinically relevant classes (Pap III – V) to discard suspicious benign or malign carcinomas.

The cytology of the vaginal smear was characterized using ~ 200 cells obtained from the lateral vaginal wall opposite the tip of cervix with a wad of cotton and transferred on a specimen holder. Fixation was performed immediately after sampling by use of Merckofix spray. Cells were classified as parabasal, intermediate or superficial cells. Gynecological cytodiagnostics was performed after staining according to the method of Papanicolau [14] and following Eq.1:

VMI (%) = (superficial cells × 1) (%) + (intermediate cells × 0.5) (%)

According to this equation, the vaginal maturation index (VMI) could be between 0 (only parabasal cells) and 100 (only superficial cells) [9]. Endometrial thickness was measured by transvaginal ultrasonic scan and recorded in mm.

Routine laboratory safety, vital signs and 12-lead ECG assessments were performed at screening and post-study only. Adverse events (AEs) were either reported spontaneously by the volunteer or observed/elicited by general questioning by a member of the study team. AEs and concomitant medication were recorded on a continuous basis.

Statistical analysis

In order to keep the global α error risk of the entire study at a level of 5%, α of the interim analysis after n = 24 was adjusted for multiple testing according to Lan & DeMets [15]. According to the procedure of Hauck et al. [16], the bounds calculated by the algorithm of Lan & DeMets were converted into the corresponding p-values and from this using the exact degrees of freedom to t-values used for the calculation of the confidence intervals (CIs). For the interim analysis after 24 subjects an α-level of 0.025 was used and, in case of completion with n = 36, a nominal α-level of 0.04 resulted. In order to obtain a power of at least 80% in case of perfect BE assuming a geometric CV of ≤ 30% for AUC_{0-t} and ≤ 50% for C_{max}, 32 subjects were calculated to complete the study using a non-sequential standard design with 90% CIs. Considering a sequential 2-stage design and a wider 92% CI after completion of all subjects, n = 36 subjects ensured a power of ≥ 80%, as increased by the interim analysis after 24 subjects. Bioequivalence was to be accepted after 24 subjects, if the 95% CIs were included by the respective acceptance ranges of 80 – 125% [17, 18, 19].

Assuming that PK parameters were log-normally distributed, the logarithms were subjected to an analysis of variance (ANOVA) for AUC_{0-t} and C_{max} for measured and net values, and $AUC_{0-\infty}$ for net values only. This model included effects accounting for the following sources of variation: "sequence", "subjects within sequences", "period", and "treatment". The effect "subjects within sequences" was considered as random, but the other effects were considered as fixed. In test and reference, descriptive statistics were calculated for E2 and E1 concentrations, derived PK parameters, and pharmacodynamic parameters, i.e. N, arithmetic mean, standard deviation (SD) with coefficient of variation, minimum (min), median, maximum (max), geometric mean and SD with coefficient of variation (gCV) and graphics as appropriate. Additionally, the ln-transformed variables of all AUC- and C_{max}-

Table 1. Demographic data of postmenopausal women included in the study (n=25) calculated as arithmetic mean ± SD, median, minimum (min) and maximum (max) values.

Parameter	Mean ± SD	Median	Min	Max
Age (years)	54.70 ± 5.35	54.00	39.00	65.00
Weight (kg)	70.89 ± 6.25	71.00	60.00	82.00
Height (cm)	165.04 ± 6.98	165.00	150.00	178.00
BMI (kg/m^2)	25.84 ± 1.98	25.80	22.0	29.00
ET (mm)	3.08 ± 0.76	3.00	2.00	4.00

BMI = Body Mass Index; ET = Endometrial thickness.

Table 2. Pre-study within-day precision and accuracy results of the GC/MS-NCI/SIM method validation applied for the quantification of estradiol (E2) and estrone (E1) in plasma.

Analyte (1 batch)	Nominal conc. values (pg/ml) (n = 6, for each conc.)	Arith. mean value ± SD (pg/ml)	Precision (CV (%))	Accuracy (deviations from nominal value, mean ± SD (%))
E2	199.8	183.32 ± 1.30	0.71	8.25 ± 0.65
	59.9	56.50 ± 0.84	1.48	5.68 ± 1.40
	12	11.45 ± 0.29	2.52	4.58 ± 2.40
E1	200	191.73 ± 5.34	2.78	4.13 ± 2.67
	60	57.67 ± 0.98	1.70	3.89 ± 1.63
	12	12.07 ± 0.36	2.95	–0.56 ± 2.97

values were described in the same way except for geometric mean and geometric SD.

Results

Subjects

A total of 25 women was included, 24 completed the study. One subject dropped out before having received any study medication. Demographic data of all 25 included subjects (full analysis set) are summarized in Table 1. Their arithmetic mean ± SD age was 54.7 ± 5.4 years, their body weight and BMI were 70.9 ± 6.25 kg and 25.84 ± 1.98 kg/m^2, respectively. At screening, gynecological examination showed cytological states classified as Pap I/II in 22 subjects and as Pap I in 2 subjects. No Pap III-V cytological states were reported. The arithmetic mean of the endometrial thickness determined at the pre-study examination was of 3.08 ± 0.76 mm (mean ± SD). With the exception of one subject, all volunteers had plasma E2 values ≤ 20 pg/ml during prestudy examination. All subjects had plasma FSH values ≥ 40 IU/l during prestudy examination and were negative for HBs-AG, anti-HCV-AB and anti-HIV-AB. All subjects were healthy with only minor deviations from normal parameters in routine safety parameters.

Some subjects showed protocol deviations considered not relevant: 1 subject had an E2 concentration slightly above the threshold, i.e., 26.9 pg/ml vs. 20 pg/ml, at prestudy examination and was included in the study; 2 other subjects were not clinically postmenopausal for ≥ 2 years, but showed postmenopausal FSH and E2 values; 3 subjects had previous and continued medication during the study, which, however, were not expected to interfere with E2V dosing.

Method validation

Within-day precision and accuracy

The results of the within-day precision and accuracy after a 6-fold determination of E2 on one single day assessed during pre-study validation showed that the mean values ± SD were 183.32 ± 1.30 pg/ml for the upper limit of quantification (QC-high: 199.8 pg/ml), 56.50 ± 0.84 pg/ml for the intermediate limit (QC-mid.: 59.90 pg/ml) and 11.45 ± 0.29 pg/ml for the lower limit of quantification (QC-low: 12.0 pg/ml). The deviations from nominal values expressed as mean ± SD (%) were calculated as 8.25% ± 0.65%, 5.68% ± 1.40%

Table 3. Pre-study between-day precision and accuracy results of the GC/MS-NCI/SIM method validation applied for the quantification of estradiol (E2) and estrone (E1) in plasma.

Analyte (3 batches)	Nominal conc. values (pg/ml) (6-fold determination on each of 3 different days)	Arith. mean value ± SD (pg/ml)	Precision (CV (%))	Accuracy (Deviations from nominal value, mean ± SD (%))
E2	199.8	186.21 ± 3.30	1.63	6.80 ± 1.52
	59.9	56.30 ± 0.89	1.59	6.01 ± 1.49
	12	11.64 ± 0.36	3.05	3.01 ± 2.96
E1	200	203.87 ± 8.42	4.13	–1.94 ± 4.21
	60	61.15 ± 2.30	3.77	–1.92 ± 3.84
	12	12.22 ± 0.36	2.95	–1.81 ± 3.00

Table 4. Pre-study recovery determinations of the GC/MS-NCI/SIM method validation applied for the quantification of estradiol (E2), estrone (E1) and the internal standard (ISTD) in plasma.

Analyte	Nominal conc. values (pg/ml) (n = 3 for each conc.)	Recovery (%) ± SD (%)
E2	250.0	86.49 ± 1.03
	81.0	86.62 ± 1.88
	10.0	73.92 ± 0.88
E1	250.0	90.07 ± 0.63
	81.0	84.41 ± 2.29
	10.0	86.64 ± 0.25
ISTD	81.0	86.96 ± 1.88

and 4.58% ± 2.40% for QC-high, QC-mid and QC-low quantification limits, correspondingly. In all the cases, the precision, expressed as the CV-values, was less than 2.52%. These results can be seen in Table 2.

In the same fashion, the results of the within-day precision and accuracy after a 6-fold determination of E1 on same conditions as described above showed that the mean values ± SD were 191.73 ± 5.34 pg/ml for the upper limit of quantification (QC-high: 200.0 pg/ml), 57.67 ± 0.98 pg/ml for the intermediate limit (QC-midrange: 60.0 pg/ml) and 12.07 ± 0.36 pg/ml for the lower limit of quantification (QC-low: 12.0 pg/ml). The deviations from nominal values expressed as mean ± SD (%), were calculated as 4.13% ± 2.67%, 3.89% ± 1.63% and –0.56% ± 2.97% for QC-high, QC-mid and QC-low quantification limits, correspondingly. In all the cases, the precision, expressed as the CV-values was less than 2.95% (Table 2).

Between-day precision and accuracy

The results of the between-day precision and accuracy after a 6-fold determination on each day of 3 different days during pre-study validation showed that the mean values ± SDs were 186.21 ± 3.30 pg/ml for the upper limit of quantification, 56.30 ± 0.89 pg/ml for the intermediate limit and 11.64 ± 0.36 pg/ml for the lower limit. The accuracy values, i.e., the deviations from nominal values, expressed as arithmetic means ± SDs (%) were calculated as 6.80% ± 1.52%, 6.01% ± 1.49% and 3.01% ± 2.96% for QC-high, QC-mid and QC-low quantification limits, correspondingly. In all the cases, the precision, expressed as the CV-values, was less than 3.05%. These results are depicted in Table 3.

Likewise, the results of the within-day precision and accuracy on the same conditions for E1 showed that the mean values ± SDs were 203.87 ± 8.42 pg/ml for the upper limit of quantification, 61.15 ± 2.30 pg/ml for the intermediate limit and 12.22 ± 0.36 pg/ml for the lower limit. The accuracy values, i.e. the deviations from nominal values, expressed as arithmetic means ± SDs (%), were calculated as –1.94% ± 4.21%, –1.92% ± 3.84% and –1.81% ± 3.00% for QC-high, QC-mid and QC-low quantification limits, correspondingly. In all the cases, the precision, expressed as the CV-values was less than 4.13% (Table 3).

Recovery

The recovery using standard samples of E2 at concentration levels of 250.0 pg/ml, 81 pg/ml and 10 pg/ml varied between 73.92% ± 0.88% and 86.62% ± 1.88%, whereas those calculated using standard samples of E1 at the same levels ranged from 86.64% ± 0.25% to 90.07% ± 0.63%. In the case of the internal standard (IS), a single standard of 81.0 pg/ml was used and the recovery was 86.96% ± 1.88% (n = 3). These results can be seen in Table 4.

Table 5. Arithmetic means of plasma concentrations ± SDs of estradiol (E2) and estrone (E1) at Day 0 (baseline, Periods I and II) and at Day 14 (Period I) for the sequences Reference → Test and Test → Reference.

Analyte	Sequence	Parameter	Period		
			I		II
			Day 0	Day 14	Day 0
Estradiol (E2)	Reference → Test	N	10/11[a]	11	8/11[c]
		Conc. ± SD (pg/ml)	LLOQ	64.01 ± 23.39	15.24 ± 7.31
	Test → Reference	N	11/13[b]	13	10/13[c]
		Conc. ± SD (pg/ml)	LLOQ	72.08 ± 30.84	23.23 ± 14.06
Estrone (E1)	Reference → Test	N	9/11[c]	11	10/11[c]
		Conc. ± SD (pg/ml)	15.21 ± 7.74	46.78 ± 28.97	19.51 ± 9.54
	Test → Reference	N	11/13[c]	13	13
		Conc. ± SD (pg/ml)	21.19 ± 7.91	49.07 ± 20.25	20.70 ± 10.36

N = Number of subjects; LLOQ = Lower limit of quantification (10 pg/ml); [a]Observed concentrations in 1 out of 11 subjects: 21.10 pg/ml; [b]Observed concentrations in 2 out of 13 subjects: 21.42 pg/ml and 76.89 pg/ml, respectively. [c]The remaining subjects presented values below LLOQ.

Specificity

The specificity of analytical method was performed by analysis of six blank plasma samples of different origin. The specificity of the method was demonstrated by selected ion monitoring (SIM) at a mass/charge ratio (m/z) of 464 for $[M]^-$ of the E1-PFBz derivative, at a m/z of 562 for $[M]^-$ of the E2-PFBz-TFA derivative and at a m/z of 566 for the internal standard E2-D4-PFBz-TFA derivative, by investigation of IS-spiked blank and standard sample and by demonstration of undisturbed peak detection in a set of samples comprising 2 samples of 2 volunteers each.

Reproducibility

The reproducibility was calculated based on 3-fold determinations of each set of standard samples during the pre-study validation period. The coefficients of variation (CV%) of the determination reproducibility for E2 and E1 were < 9.1% and < 7.9%, respectively.

Stability

The stability determination was performed using E2/E1 plasma samples storaged at –20 °C for 18 days and upon repeated freezing and thawing cycles as well as with drug/metabolite samples in extracts storaged at room temperature for 3 days and at –20 °C for 6 days. The E2/E1 concentrations of the samples used for the stability determination ranged from 10 to 250 pg/ml. In all the cases, the deviation with reference to the measured values remained within the accepted range of the accuracy of the method, i.e. within 15% of the nominal values.

Pharmacokinetics and bioequivalence

In order to facilitate comparisons of the plasma concentrations at baseline, arithmetic means ± SDs of estradiol (E2) and estrone (E1) over the time following different sequences of administration were presented by period taking into account the levels at the first day of administration (Day 0) of both periods and those at an intermediate point (Day 14) of Period I as shown in Table 5. Nearly all female subjects showed arithmetic mean baseline E2 concentrations below LLOQ (< 10 pg/ml) at –48, –24, –12 and 0 h, except for 1 subjects with reference product and 2 subjects with test product as their first treatments. However, 14 days after the first dose of the reference product and immediately before the treatment with test product, the subjects showed arithmetic mean concentrations of 64.01 ± 23.39 pg/ml (11 subjects) and 15.24 ± 7.31 pg/ml (8 out of 11 subjects) for E2, respectively (Table 5). 14 days after the first dose of the test product and immediately be-

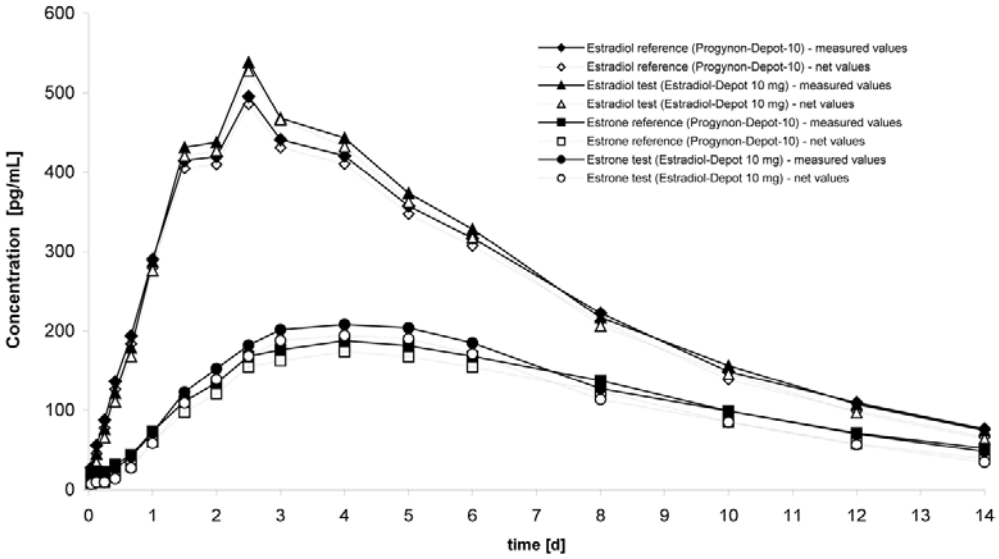

Figure 1. Arithmetic mean plasma concentrations of estradiol (E2) and estrone (E1) after i.m. administration of reference and test products in postmenopausal women (n = 24 subjects, measured vs. net values).

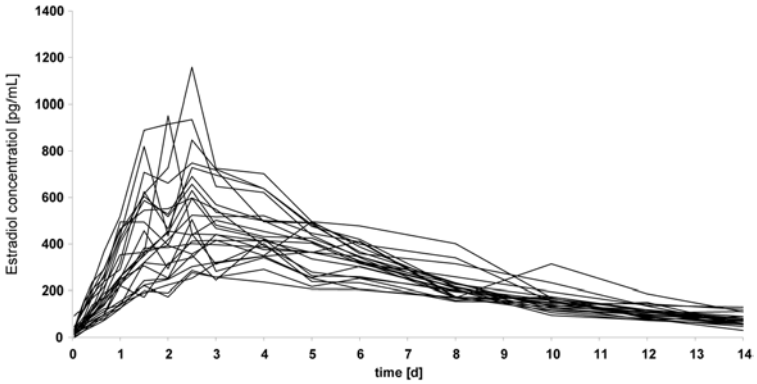

Figure 2. Individual measured plasma concentrations of estradiol in test product (Estradiol-Depot 10 mg) after i.m. administration in postmenopausal women (n = 24).

fore the treatment with reference product, the mean E2 concentrations were 72.08 ± 30.84 pg/ml (13 subjects) and 23.23 ± 14.06 pg/ml (10 out of 13 subjects), respectively (Table 5). Comparing the arithmetic mean concentrations of E2 observed in Period I and II at Day 0 following both treatment sequences, the results clearly indicated a carryover effect of the i.m. administration of E2V on the plasma levels of E2, i.e., the presence of E2 increased at a time point, at which such increase was not expected. Therefore, this observation demonstrated that the time assigned for the washout period, i.e., 4 weeks, was insufficient to allow the return of the E2 concentrations to baseline levels in the subjects.

For E1, 9 out of 11 subjects showed an arithmetic mean concentration of 15.21 ± 7.74 pg/ml prior to their first dose of the reference product. Eleven out of 13 subjects showed an arithmetic mean concentration of 21.19 ± 7.91 pg/ml prior to their first dose of the test product. 14 days post-dose and immediately prior to the second treatment (Period II, Day 0), those values were calculated as 46.78 ± 28.97 pg/ml (11 subjects) and 19.51 ± 9.54 pg/ml (10 out of 11 subjects) when the reference product was used in the first period. When the test product was used in the first period, the arithmetic mean concentrations of E1 were calculated as 49.07 ± 20.25 pg/ml on Day 14 and 20.70 ± 10.36 pg/ml on Day 0 of Period II in identical number of subjects, i.e. 13. Thus, independently from the treatment, such PK data may indicate that, with slight differences, a return into baseline values was observed for E1 after a washout period of 4 weeks (Table 5).

The mean plasma concentration vs. time profiles of the E2 and E1 derived from measured and net values after i.m. administration of reference (Progynon Depot®) and test (Estradiol-Depot® 10 mg) products can be seen in Figure 1. Due to the low baseline concentrations, curves for measured concentrations and for net concentrations were nearly identical for both analytes. Furthermore, the plasma concentrations of the parent compound E2 were 2- to 3-fold higher than the metabolite E1.

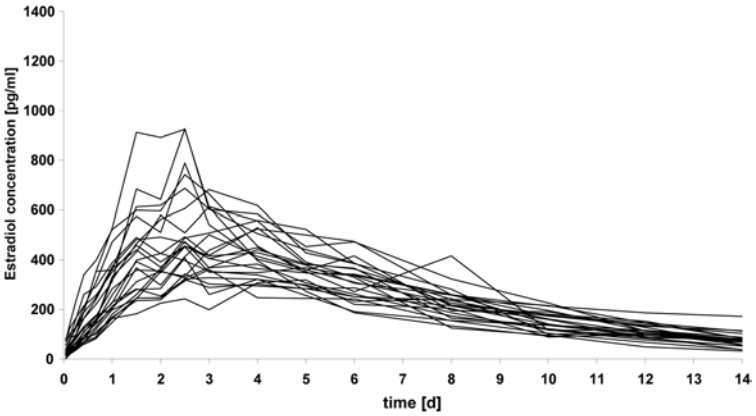

Figure 3. Individual measured plasma concentrations of estradiol in reference product (Progynon Depot 10 mg) after i.m. administration in postmenopausal women (n = 24).

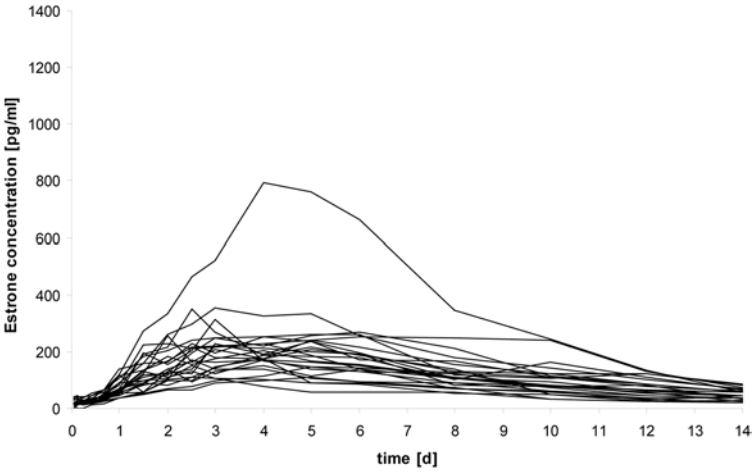

Figure 4. Individual measured plasma concentrations of estrone in test product (Estradiol-Depot 10 mg) after i.m. administration in postmenopausal women (n = 24).

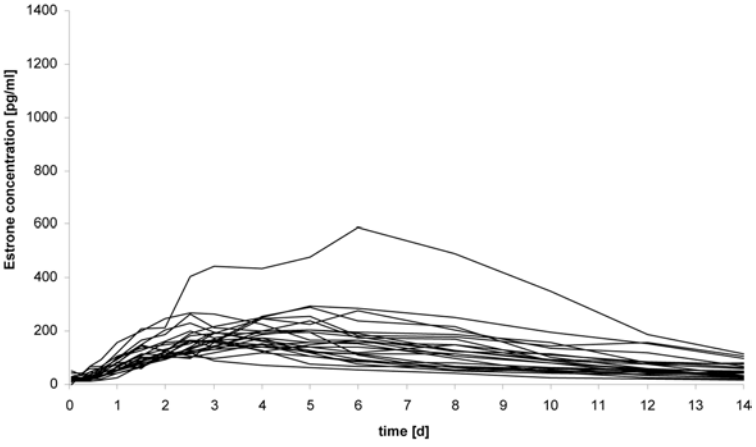

Figure 5. Individual measured plasma concentrations of estrone in reference product (Progynon Depot 10) after i.m. administration in postmenopausal women (n = 24).

After the administration of both products the profiles of both analytes (E2 and E1) were very similar. Mean E2 peak concentrations of ~ 500 pg/ml were detected at ~ 2.5 days for both products, whereas mean E1 peak concentrations of ~ 200 pg/ml could be determined after 3 days in both cases. After 14 days, E2 and E1 concentrations of ~ 100 pg/ml or less were observed.

The interindividual variability of the pharmacokinetic profiles of E2 after test and reference can be deduced from Figures 2 and 3, respectively. No marked differences in profiles are observed after the administration of either test or reference product. Peak concentrations in a range from ~ 350 pg/ml to ~ 1,000 pg/ml were observed within 1 – 3 days. Only in 1 subject, a peak concentration higher than 1,000 pg/ml could be observed after the administration of the test product. After 14 days concentrations below 200 pg/ml could be detected in all subjects.

For E1 after test and reference, peak concentrations in a range from ~ 150 pg/ml and 400 pg/ml are observed between 3 and 5 days and concentrations generally lower than 100 pg/ml could be seen after 14 days. For details see Figures 4 and 5. One subject presented a different E1 profile with concentrations higher than those aforementioned following both administration of test and reference products indicating a slightly diverging metabolic pattern.

From evaluation of the measured E2 concentrations, geometric mean C_{max}-values were calculated as 543.5 pg/ml (gCV = 39%) and as 505.7 pg/ml (gCV = 33%) for test and for reference, respectively. Affiliated AUC_{0-t}-values for test amounted to 84,734 pg × h/ml (gCV = 22%) for test and to 82,660 pg × h/ml (gCV = 21%) for reference. The aforementioned results are shown in Table 6.

For the measured E1 concentrations, geometric mean C_{max}-values were calculated as 219.0 pg/ml (gCV = 44%) and 204.9 pg/ml (gCV = 35%) for test and reference, respectively. AUC_{0-t}-values amounted to 38,950 pg × h/ml (gCV = 42%) for test and to 37,159 pg × h/ml (gCV = 42%) for reference (Table 6).

E2 net concentrations derived from subtractive baseline correction were used for calculation of geometric mean values of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ and resulted correspondingly in 533.5 pg/ml (gCV = 39%), 81,285 pg

Table 6. Summary of pharmacokinetic parameters (measured values) of estradiol (E2) and estrone (E1) determined after IM administration of Estradiol-Depot 10 mg® (test) and Progynon Depot-10® (reference) in 24 postmenopausal women.

Analyte	Parameter	Treatment	Geometric mean	Geometric CV (%)	Medians (Min – Max)
Estradiol (E2)	C_{max} (pg/ml)	Test	543.5	39	–
		Reference	505.7	33	–
	t_{max} (h)	Test	–	–	60.0 (48 – 120)
		Reference	–	–	60.0 (36 – 120)
	AUC_{0-t} (pg × h/ml)	Test	84 733.8	22	–
		Reference	82 660.4	21	–
	$t_{½}$ (h)	Test	–	–	88.3 (39.38 – 170.67)
		Reference	–	–	89.9 (52.82 – 200.20)
Estrone (E1)	C_{max} (pg/ml)	Test	219.0	44	–
		Reference	204.9	35	–
	t_{max} (h)	Test	–	–	96.0 (48 – 144)
		Reference	–	–	96.0 (48 – 144)
	AUC_{0-t} (pg × h/ml)	Test	38 949.9	42	–
		Reference	37 159.4	42	–
	$t_{½}$ (h)	Test	–	–	101.7 (54.8 – 161.24)
		Reference	–	–	89.9 (65.63 – 277.24)

Table 7. Summary of pharmacokinetic parameters (net values) of estradiol (E2) and estrone (E1) determined after IM administration of Estradiol-Depot 10 mg® (test) and Progynon Depot-10® (reference) in 24 postmenopausal women.

Analyte	Parameter	Treatment	Geometric mean	Geometric CV (%)	Medians (min – max)
Estradiol (E2)	C_{max} (pg/ml)	Test	543.5	39	–
		Reference	505.7	33	–
	t_{max} (h)	Test	–	–	60.0 (48 – 120)
		Reference	–	–	60.0 (36 – 120)
	AUC_{0-t} (pg × h/ml)	Test	84 733.8	22	–
		Reference	82 660.4	21	–
	$t_{½}$ (h)	Test	–	–	85.7 (29.58 – 148.17)
		Reference	–	–	80.9 (52.82 – 171.63)
Estrone (E1)	C_{max} (pg/ml)	Test	219.0	44	–
		Reference	204.9	35	–
	t_{max} (h)	Test	–	–	96.0 (48 – 144)
		Reference	–	–	96.0 (48 – 144)
	AUC_{0-t} (pg × h/ml)	Test	38 949.9	42	–
		Reference	37 159.4	42	–
	$t_{½}$ (h)	Test	–	–	83.1 (44.58 – 143.36)
		Reference	–	–	85.1 (54.02 – 212.75)

× h/ml (gCV = 22%) and 90,511 pg × h/ml (gCV = 19%) for test, and 494.8 pg/ml (gCV = 33%), 79,295 pg × h/ml (gCV = 21%), and 88,706 pg × h/ml (gCV = 21%) for reference. These results are depicted in Table 7.

The corresponding geometric mean C_{max}-, AUC_{0-t}-, and $AUC_{0-\infty}$-values for baseline-corrected net E1 values were 204.6 pg/ml (gCV = 47%), 33,943 pg × h/ml (gCV = 49%) and 38,641 pg × h/ml (gCV = 48%) for test and 191.1 pg/ml (gCV = 37%), 32,397 pg × h/ml (gCV = 48%) and 37,850 pg × h/ml (gCV = 50%) for reference (Table 7).

Furthermore, medians with minimum and maximum values of t_{max} for E2 as measured or net values were 60 h (48 – 120 h) and 60 h (36 – 120 h), for test and reference, respectively. In the case of E1, the corresponding medians with minimum and maximum values of t_{max} were identical, i.e. 96.0 h (48 – 144 h), after test and reference. However, due to the formulation characteristics, which result in a

Table 8. Point estimates (PEs) and confidence intervals (CIs, 95%) of the test/reference (T/R) mean ratios of parameters C_{max} and AUC_{0-t} calculated from measured and net values and determined by ANOVA.

Analyte	Values	Parameter	PE (T/R) (%)	95% CI	
				lower (%)	upper (%)
Estradiol (E2)	Measured	C_{max} (pg/ml)	107.3	93.9	122.5
		AUC_{0-t} (pg × h/ml)	102.5	94.5	111.2
	Net	C_{max} (pg/ml)	107.4	94.2	122.5
		AUC_{0-t} (pg × h/ml)	102.1	94.4	110.4
Estrone (E1)	Measured	C_{max} (pg/ml)	106.9	94.4	121.0
		AUC_{0-t} (pg × h/ml)	105.0	95.7	115.2
	Net	C_{max} (pg/ml)	106.9	93.8	121.8
		AUC_{0-t} (pg × h/ml)	104.6	94.1	116.3

pronounced plateau phase, this parameter is of limited value to establish differences between products (Table 6).

The medians with minimum and maximum values of the net apparent terminal elimination half-lives ($t_{1/2}$) of E2 were calculated as 85.7 h (29.58 – 148.17 h) and 80.9 h (52.82 – 171.63 h) after test and reference, respectively. For E1 the corresponding $t_{1/2}$-values were calculated as 83.1 h (44.58 – 143.36 h) and 85.1 h (54.02 – 212.75 h) after test and reference, correspondingly (Table 6). These results showed that practically no differences in the $t_{1/2}$-values of both analytes independently form the treatment.

Using measured values, for E2 the point estimates (PEs) of the Test/Reference (T/R) mean ratios of the variables C_{max} and AUC_{0-t} were 107.3% (95% CI = 93.9 – 122.5%) and 102.5% (95% CI = 94.5 – 111.2%), respectively, whereas for E1 those of C_{max} and AUC_{0-t} were 106.9% (95% CI = 94.4 – 121.0%) and 105.0% (95% CI = 95.7 – 115.2%), respectively. For net values of E2, the point estimates (PEs) of the Test/Reference (T/R) mean ratios of the variables C_{max} and AUC_{0-t} were 107.4% (95% CI = 94.2 – 122.5%) and 102.1% (95% CI = 94.4 – 110.4%), respectively, whereas those of C_{max} and AUC_{0-t} for E1 were 106.9% (95% CI = 93.8 – 121.8%) and 104.6% (95% CI = 94.1 – 116.3%), respectively. These results are presented in Table 8.

In general, the formulation related PK parameters show no remarkable differences between both products. The comparisons for BE assessment were performed using measured as well as net values. The point estimates and 95% confidence intervals for AUC_{0-t} and C_{max} after administration of the test and reference formulation clearly indicate bioequivalence of both products as confidence intervals of all parameters are within the acceptance limits (80 – 125%). Thus, the study could be stopped after the interim analysis of 24 subjects without further enrolment up to n = 36.

Pharmacodynamics and safety

Vaginal maturation index

The results of the changes in the vaginal maturation index (VMI) after treatment comparing median values (min-max) derived on Days –2, 8, 14 and 21 days can be seen in Table 9. In this table, the median values corresponding to Day –2 represent the baseline values. Following the treatment with the test product in Period I, a remarkable increase in VMI could be observed 8 days after the first i.m. injection as compared with the baseline, i.e. 93.50% (76.00 – 97.00%) vs. 46.75% (4.00 – 88.00%). Thereafter, the VMI remained practically unchanged as demonstrated by the values observed after 14 days, i.e. 94.50% (68.00 – 98.00%). However, a slight decrease was seen after 21 days, i.e. 84.50% (67.00 – 97.50%). For the second period, the observed baseline median value was 94.50% (86.00 – 97.00%) and even after 21 days practically no modification of this value was observed, i.e. 96.00% (95.50 – 98.50%).

Also after reference in Period I, a remarkable increase in VMI could be observed 8 days after the first i.m. injection as compared with the baseline, i.e. 86.00% (53.00

Table 9. Vaginal maturation indices (%) expressed as medians with minimum and maximum values at Days –2, 8, 14 and 21 after Periods I and II separated for test and reference treatment

	Day	–2		8		14		21	
	Period	I	II	I	II	I	II	I	II
Test	N	13	11	13	11	13	11	13	11
	Median (%)	46.75	94.50	93.50	96.00	94.50	96.50	84.50	96.00
	Min (%)	4.00	86.00	76.00	54.00	68.00	83.50	67.00	95.50
	Max (%)	88.00	97.00	97.00	98.50	98.00	98.50	97.50	98.50
Reference	N	12*	13	11	13	11	13	11	13
	Median (%)	60.25	94.00	86.00	96.50	92.75	94.50	94.50	96.00
	Min (%)	4.00	83.50	53.00	92.00	64.50	54.50	75.50	83.50
	Max (%)	88.50	97.50	98.00	98.50	98.50	98.00	98.00	98.50

*Dropout.

Table 10. Endometrial thicknesses (mm) expressed as medians with minimum and maximum values at Days –2, 8, 14 and 21 after Periods I and II separated for test and reference treatment.

	Day	–2		8		14		21	
	Period	I	II	I	II	I	II	I	II
Test	N	13	11	13	11	13	11	13	11
	Median (mm)	3.00	4.00	8.00	8.00	7.00	8.00	7.00	7.00
	Min (mm)	2.00	3.00	5.00	4.00	6.00	4.00	4.00	4.00
	Max (mm)	5.00	6.00	9.00	13.00	16.00	13.00	11.00	12.00
Reference	N	12*	13	11	13	11	13	11	13
	Median (mm)	3.00	5.00	7.00	8.00	7.00	9.00	5.00	7.00
	Min (mm)	2.00	4.00	5.00	5.00	4.00	5.00	4.00	3.00
	Max (mm)	4.00	50.00	9.00	17.00	9.00	18.00	9.00	17.00

*Dropout.

– 98.00%) vs. 60.25% (4.00 – 88.50%). The VMI presented a slight increase after 14 days, i.e. 92.75% (64.50 – 98.50%), and thereafter remained practically unchanged as demonstrated by the values observed after 21 days, i.e. 94.50% (75.50 – 98.00%). For the second period, the observed baseline median value was 94.00% (83.50 – 97.50%) and after 21 days no modification of this value was observed, i.e. 96.00% (83.50 – 98.50%).

The data demonstrate that maturation index increased in a comparable way after test as well as after reference in Period I and remained nearly unchanged for both products during washout phase and after the second treatment in Period II. This latter observation may be an indicative of a carryover effect probably induced by remaining amount of E2 due to an insufficient washout phase after the Period I. In general, both formulations favored the vaginal maturation after a short period, i.e. 8 days after administration, and were capable to maintain this effect during at least over 21 days.

Endometrial thickness

Endometrial thickness (ET) after test and reference treatment comparing values derived on Days –2, 8, 14 and 21 days are shown in Table 10. The treatment with the test product in Period I, also exhibited a noticeable increase 8 days after the administration as compared with the baseline value (Day –2), i.e., 8.00 mm (5.00 – 9.00 mm) vs. 3.00 mm (2.00 – 5.00 mm). After 14 days, the ET-value was kept at approximately the same level, i.e., 7.00 mm (6.00 – 16.00 mm) after 14 days and no further modification of this latter was observed after 21 days, i.e., 7.00 mm (4.00 – 11.00 mm). For Period II, the observed median baseline value was 4.00 mm (3.00 – 6.00 mm) and increased to 8.00 mm (4.00 – 13.00 mm) after 8 days. The median ET-value remained exactly at the same level after 14 days, i.e.

8.00 mm (4.00 – 13.00 mm), and decreased to 7.00 mm (4.00 – 12.00 mm) after 21 days.

The treatment with the reference product in Period I also exhibited an increase 8 days after the administration as compared with the median baseline value (Day –2), i.e., 7.0 mm (5.00 – 9.00 mm) vs. 3.00 mm (2.00 – 4.00 mm). The median ET was identical after 14 days, i.e., 7.00 mm (4.00 – 9.00 mm), and decreased to 5.00 mm (4.00 – 9.00 mm) after 21 days. For Period II, the observed median ET baseline value was 5.00 mm (4.00 – 50.00 mm). It increased to 8.00 mm (5.00 – 17.00 mm) after 8 days and again to 9.00 mm (5.00 – 18.00 mm) after 14 days. However, it decreased to 7.00 mm (3.00 – 17.00 mm) after 21 days.

The data demonstrate that ET increased in a comparable way after test and after reference in Period I. Overall, the median ET baseline values were higher in Period II compared with those of the Period I independently from the treatment. Like in the description of the results of the VMI, the presence of higher median baseline values at the beginning of the Period II may also suggest that the time used for the washout phase was insufficient to eliminate remain amounts of E2 inducing endometrial growth. In general, both formulations favored the increase in ET after a short period, i.e., 8 days after administration, and supported the prevalence this effect at least over 21 days.

Adverse events (AEs)

87% (21/24) of the volunteers reported an AE during the study. A decline of number of AEs per period with progression of the study was observed (59 in Period 1, 21 in Period 2). 59% (49/82) of the AEs were assessed as definitely, probably or possibly drug related and occurred in 18 out of 24 volunteers. 30 AEs were assessed as being unlikely or not related to the study drug.

Most of the AEs, 72% (59/82) of the total and 84% (41/49) of those regarded as drug related, occurred in Period 1 of the study. 16% (8/49) of drug-related AEs occurred in Period 2. Therefore, also the number of related AEs declined with progression of the study. Three types of AEs, namely abdominal swelling (n = 5), swelling of outer genitals (n = 6) and tighten of breasts (n = 5) accounted for 33% of the related AEs (16/48).

After test, a total of 34 AEs, 25 of them assessed as study drug related, was reported in 16 out of 24 (67%) volunteers. After reference, a total of 43 AEs, 22 of them assessed as study drug related, was reported. Additionally, 5 AEs started prior to the first drug administration (not related). The most frequent AE was headache (n = 19, in 12 volunteers). 13 of the 24 volunteers took concomitant medications. None of these medications was expected to have an effect on the results of the study. 14 volunteers (58%) reported an AE in both study periods. There were no serious adverse events reported.

Discussion

So far, with regard to pharmacokinetics and bioavailability of E2 and E1 after intramuscular administration of E2V, only few pilot studies with small sample sizes of subjects have been published. Oriowo et al. [7] assessed the E2 and E1 plasma concentrations following i.m. administration of E2V in 9 healthy young women (ages 20 – 35 years). Düsterberg et al. determined the E2 plasma levels after i.m. administration of E2V in 3 postmenopausal women as well as the levels of E2, E1, E1-conjugates, estriol and estriol conjugates after i.m. administration of E2V in 2 ovariectomized women [8, 20]. Finally, Göretzlehner et al. [21] characterized also E2 and E1 profiles after i.m. administration of E2V in 17 women. However, contrary to the present study, none of these aforementioned studies was carried out with simultaneous assessment of surrogates for efficacy in postmenopausal women, e.g. endometrial thickness or vaginal maturation index. For these reasons, new studies with sufficient number of subjects and simultaneous assessment of pharmacodynamic parameters were required in order to allow a better insight in the pharmacokinetics and pharmacodynamics of E2V when administered as intramuscular injection intended for a 4 weeks dosing interval to postmenopausal women.

In the context of the present study, the analytical method developed for quantitation of E2 and E1 with a lower limit of quantitation of 10 pg/ml obviously showed sufficient sen-

sitivity for adequate profiling of both analytes after intramuscular administration of 10-mg E2V. The GC/MS-NCI/SIM method demonstrated adequate within-day and between-day accuracy as deviations from nominal values indicated in Tables 2 and 3 were less than 15% in the corresponding study sets. The method also showed adequate within-day and between-day precision, as CVs in study sets indicated in same tables were always less than 15%. The method presented recoveries for E2 and E1, which were in good agreement with the recoveries of the internal standard at three different nominal concentrations.

In terms of the concentrations of E2 and E1, the pharmacokinetic data from the present study are in good agreement with those of earlier studies performed in postmenopausal women study with a single i.m. dose of 4-mg E2V [8] as well as with those performed with healthy women with a single i.m. dose of 5 mg [7]. In the aforementioned studies, higher concentrations of E2 compared to those of E1 were always observed. This difference can be explained as a consequence of the i.m. route of administration, at which, in contrast to oral administration, E1 concentrations are lower than E2 levels due to the missing first-pass elimination.

Compared to the data from Düsterberg et al. [8] with a 4-mg i.m. dose of E2V, administered to 2 subjects only, the geometric mean C_{max}-values of E2 of around 500 pg/ml after a 10-mg dose in the present study were close to the mean value reported for the 4-mg dose, i.e. 440 pg/ml. Furthermore, t_{max}-values of around 3 days were comparable in both studies. Here, it must be pointed out that the formulation administered in the study from Düsterberg et al. used the same vehicle composition as that described in this study, i.e. benzyl benzoate and castor oil.

Compared to the data from Oriowo et al. [7] with a 5-mg i.m. dose of E2V, administered to 30 healthy women, the observed geometric mean C_{max}-values of E2 and E1 in both studies did not markedly differ, i.e. 500 pg/ml vs. 600 pg/ml for E2 and 200 pg/ml vs. 250 pg/ml for E1. The t_{max}-values of E2 and E1 for test and reference products observed in this study were ~ 2.91 and 3.91 days, respectively. However, in the study of Oriowo et al. the corresponding t_{max}-values of E2 and E1 were reported after 2 and 3 days, respectively.

This slight difference in the release may be attributed to the formulation composition, because the formulation used by Oriowo et al. [22] was composed of arachis oil only, which shows a lower kinematic viscosity than that of the castor oil at 37.8 °C (42 cSt vs. 259 cSt). This property of the arachis oil may have permitted as faster diffusion and release of E2V; however this explanation is speculative and not based on systematic investigations.

In this study both, the measured values of E2 and E1 as well as the baseline corrected net values have been considered. Measured geometric mean AUC_{0-t}-values of E2 in the current study have been determined as 84.7 and 82.7 ng×h/ml (test and reference), and thus were also similar to 66.2 and 88.9 ng × h/ml E2 observed in the pilot study published by Kuhnz et al. [6] with 2 females only. Düsterberg et al. [8] reported conjugated E1 data only and in the study carried out by Oriowo et al. [7] no E1 values were reported, so that no further comparisons are possible.

In another earlier study with only sparse measurements performed by Göretzlehner et al. [21] after i.m. administration of 5-mg E2V to 17 postmenopausal women, the highest mean concentrations were observed on Day 5, amounting to 272 pg/ml for E2 and 447 pg/ml for E1 and resulting in an E1/E2 ratio of 1.65. Such a finding is in contrast to other data in literature and to the present study, at which E1/E2 ratios of 0.38 for test and reference products were calculated. E1/E2 ratios above 1 are well known for oral administration of E2V due to a pronounced first pass effect, thus such a metabolic ratio is mechanistically implausible after i.m. administration. It is difficult to judge what might be the reason for this observation as information about population characteristics is sparse. These authors used enzyme immunoassays for E2 and E1 determination, while a GC/MS-NCI/SIM method was used in the present study. Thus, the most probable explanation is that the lesser selectivity of the assay might have resulted in an overestimation of E1 concentrations. This assumption is supported by the fact that using the data from Düsterberg et al. [8], high E1/E2 ratios for C_{max} and AUC could be calculated when conjugated E1 (and not free E1) concentrations were considered. The aforementioned factors may at least partially ex-

plain the differences in the metabolic ratios observed in these two studies. Furthermore, the mean age of postmenopausal women in the present study was 13 years lower, but the measured baseline E2 and E1 concentrations were always significantly lower.

The primary aim of this study was to assess bioequivalence of the two products based on baseline corrected E2 and E1 concentrations. According to the net values of the PK parameters of E2, the bioequivalence criteria were fulfilled with 95% CIs of 94.4%-110.4% for AUC_{0-t} and 94.2 – 122.5% for C_{max}, respectively. The same could be observed with the net PK values of E1, i.e. 95% CIs of 94.1 – 116.3% for AUC_{0-t} and 93.8 – 121.8% for C_{max}, respectively. Point estimates for AUC-values were generally close to 100%, for C_{max}-values a trend to slightly higher ratios with PE around 106 – 107% still showed high similarity of both products. PK parameters of E2 and E1 derived from measured values and from net values were highly similar. Considering these data, bioequivalence of both products could unambiguously be demonstrated.

This is the first study showing the time course of the pharmacodynamic parameters endometrial thickness and maturation index after intramuscular administration of E2V to postmenopausal women. It is well known that estrogens stimulate proliferation of the endometrium and that an uncontrolled proliferation may lead to hyperplasia and endometrial carcinoma [23, 24]. So far, PD data relating to endometrial response to HRT were mainly confined to estrogen/progestin combination therapies of longer duration [25, 26], but no comparable data were available for E2V alone, in particular not for E2V after i.m. administration. However, data from the present study indicated that the E2 concentration levels reached were more than sufficient to stimulate the growth of the endometrial cells.

Both treatments led to an increase in ET and VMI to a similar extent as demonstrated by other estrogen stimulation studies. The increase in endometrial thickness from 3 mm to ~ 8 mm observed between Days 8 and 21 after a single i.m. dose of 10-mg E2V, noticed already in the first period, was comparable to that observed after daily p.o. treatment with 0.06 mg ethinylestradiol after 14 days, at which an increase from 2 to 7 mm was observed [27]. The data suggest that already within the 21 days of each observation period a decline in ET can be observed. However, no complete return to baseline occurs within 4 weeks of washout and in the subsequent second treatment period and even further slight increases to 9 mm could be detected without observing product-related differences.

The vaginal maturation indices observed in this study were comparable or slightly higher than those observed after the treatment with daily oral doses of 0.3 mg of conjugated estrogens (87 – 92% vs. < 50% on Day 14) [9]. Maximum is nearly reached after 8 days and the effect continues beyond the dosing interval of 4 weeks. A slight trend to even higher values is observed in the second period and again, no product related differences were observed. The similarity of the PD effects of both products was to be expected as both products were clearly bioequivalent.

In the context of this study, it could be seen that the plasma levels of E1 returned to baseline after the washout period, whereas those of E2 did not. Then again, in terms of physiology, it has been reported that among estrogens, E2 exhibits the highest affinity towards nuclear cellular fractions of human endometrium, myometrium, and vagina, and accumulates on those tissues. In comparison with E2, E1 shows essentially lower affinity and it does not accumulate on them [28]. These facts may indicate that the elevated plasma levels of E2 detected in this study were the only responsible for the PD effects described, i.e. increase in VMI as well as in ET.

In addition, the estrogen stimulation in this study was also confirmed by several of the adverse events observed, i.e., abdominal swelling, swelling of outer genitals and tighten of breasts. The aforementioned symptoms have been reported in other studies conducted in postmenopausal women under hormone replacement therapies [29, 30].

Conclusion

Using measured as well as net values of E2 and E1 for calculation of C_{max} and AUC_{0-t}, bioequivalence of both estradiol valerate drug formulations, i.e. Estradiol-Depot 10 mg®

(test, Jenapharm) and Progynon Depot-10® (reference, Schering AG) could clearly be demonstrated. The endometrial thickness and the vaginal maturation index showed a direct response within the first treatment period and indicated that within 4 weeks no complete return to baseline occurs. Duration of effect was more pronounced for maturation index compared to endometrial thickness. The magnitude of effect was similar for both products and comparable to other HRTs. The adverse event pattern with abdominal swelling, swelling of outer genitals and tighten of breasts was in good accordance with the known side effect profile of HRT.

Acknowledgment

This study was performed by SocraTec R&D and was supported by Jenapharm. We like to thank Dr. Martin Brandt and Dr. Eva Maria Jauker-Blaufuß for their kind support during the gynecological and cytological analysis. We thank the bioanalytical laboratory Pharmakin GmbH and the company Quintiles ClinData for the support during the sample analysis and pharmacokinetic calculations, respectively. Furthermore, we thank Dr. Karl Ludwig Rost and Dr. Ramón Villalobos Hernández for their support in the preparation of this manuscript.

References

[1] *Kuhl H.* Pharmacology of estrogens and progestogens: influence of different routes of administration. Climacteric. 2005; *8 (Suppl 1):* 3-63. doi:10.1080/13697130500148875 PubMed

[2] *Kuhl H.* Pharmacokinetics of oestrogens and progestogens. Maturitas. 1990; *12:* 171-197. doi:10.1016/0378-5122(90)90003-O PubMed

[3] *Moskowitz D.* A comprehensive review of the safety and efficacy of bioidentical hormones for the management of menopause and related health risks. Altern Med Rev. 2006; *11:* 208-223. PubMed

[4] *Gräser T, Hoffman H, Zimmerman H, Oettel M.* Lafamme®: A new oral preparation for continuous combined hormone replacement therapy in postmenopausal women. Drugs Today (Barc). 2001; *37 (Suppl. G):* 12–27.

[5] *Lobo RA, Cassidenti DL.* Pharmacokinetics of oral 17 β-estradiol. J Reprod Med. 1992; *37:* 77-84. PubMed

[6] *Kuhnz W, Gansau C, Mahler M.* Pharmacokinetics of estradiol, free and total estrone, in young women following single intravenous and oral administration of 17ß-estradiol. Drug Res. 1993; *43:* 966-973.

[7] *Oriowo MA, Landgren BM, Stenström B, Diczfalusy E.* A comparison of the pharmacokinetic properties of three estradiol esters. Contraception. 1980; *21:* 415-424. doi:10.1016/S0010-7824(80)80018-7 PubMed

[8] *Düsterberg B, Schmidt-Gollwitzer M, Hümpel M.* Pharmacokinetics and biotransformation of estradiol valerate in ovariectomized women. Horm Res. 1985; *21:* 145-154. doi:10.1159/000180039 PubMed

[9] *Marx P, Schade G, Wilbourn S, Blank S, Moyer DL, Nett R.* Low-dose (0.3 mg) synthetic conjugated estrogens A is effective for managing atrophic vaginitis. Maturitas. 2004; *47:* 47-54. doi:10.1016/S0378-5122(03)00240-8 PubMed

[10] *Sit ASY, Modugno F, Hill LM, Martin J. Weissfeld JL.* Transvaginal ultrasound measurement of endometrial thickness as a biomarker for estrogen exposure. Cancer Epidemiol Biomarkers Prev 200; *13(9):* 1459-1465.

[11] *Lichtenberg V, Schulte-Baukloh A, Lindner C, Brändle W.* Die Bestimmung von 17-β-Östradiol (E2) im Serum von Frauen unter einer Östrogen-Substitutionstherapie liefert diskrepante Ergebnisse mit verschiedenen Immunoassay-Kits. Lab Med. 1992; *16:* 412-418.

[12] *Shah VP, Midha KK, Dighe S, McGilveray LJ, Skelly JP, Yacobi A, Layloff T, Viswanathan CT, Cook CE, McDowall RD, Pittman KA, Spector S.* Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies. J Pharmacol Sci. 1992; *81:* 309-312. doi:10.1002/jps.2600810324

[13] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). Guidance for Industry: Bioanalytical Method validation; 2001.

[14] *Soost HJ, Baur S.* Gynäkologische Zytodiagnostik. Stuttgart – New York: Georg Thieme; 5th edition, 1990.

[15] *Lan G, DeMets D.* Discrete sequential boundaries for clinical trials. Biometrika. 1983; *70:* 659-663. doi:10.2307/2336502

[16] *Hauck WW, Preston PE, Bois FY.* A group sequential approach to crossover trials for average bioequivalence. J Biopharm Stat. 1997; *7:* 87-96. doi:10.1080/10543409708835171 PubMed

[17] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). Guidance for Industry: Bioavailability and Bioequivalence for Orally Administered Drug Products – General Considerations; 2003.

[18] *Diletti E, Hauschke D, Steinijans VW.* Sample size determination for bioequivalence assessment by means of confidence intervals. Int J Clin Pharmacol Ther Toxicol. 1992; *30 (Suppl. 1):* 51-58. PubMed

[19] *Diletti E, Hauschke D, Steinijans VW.* Sample size determination: extended tables for the multiplicative model and bioequivalence ranges of 0.9 to 1.11 and 0.7 to 1.43. Int J Clin Pharmacol Ther Toxicol. 1992; *30 (Suppl. 1):* 59-62.

[20] *Düsterberg B, Wendt H.* Plasma levels of dehydroepiandrosterone and 17 β-estradiol after intramuscular administration of Gynodian-Depot in 3 women. Horm Res. 1983; *17:* 84-89. doi:10.1159/000179680 PubMed

[21] *Göretzlehner G, Bergmann G, Angelow K, Ackermann K, Kliem O, Bieck E, Golbs S.* Pharmakokinetik von Estron, Estradiol, FSH, LH und Prolaktin nach intramuskulärer Applikation von 5 mg Estradiolvalerat. J Menopause. 2002; *9:* 52-56.

[22] http://www.csgnetwork.com/sgvisc.html (accessed April 25, 2011).

[23] *Brinton LA, Hoover RN.* The endometrial cancer collaborative group. Estrogen replacement therapy and cancer risk: unresolved issues. J Obstet Gynaecol. 1993; *81:* 165-171.

[24] *Deligdisch L, Holinka CF.* Endometrial carcinoma: two diseases? Cancer Detect Prev. 1987; *10:* 237-246. PubMed

[25] *Whitehead MI, Townsend PT, Pryse-Davies J, Ryder TA, King RJ, King RJB.* Effects of estrogens and progestins on the biochemistry and morphology of the postmenopausal endometrium. N Engl J Med. 1981; *305:* 1599-1605. doi:10.1056/NEJM198112313052701 PubMed

[26] *Woodruff JD, Pickar JH; The Menopause Study Group.* Incidence of endometrial hyperplasia in postmenopausal women taking conjugated estrogens (Premarin) with medroxyprogesterone acetate or conjugated estrogens alone. Am J Obstet Gynecol. 1994; *170:* 1213-1223. PubMed

[27] *Heger-Mahn D, Entezami M, Schütt B, Kuhnz W, Richert K, Lübbert H.* Oestrogenenous Effects of 0.06 mg Ethinylestradiol over 14 Days in Postmenopausal Women. Geburtshilfe Frauenheilkd. 1996; *56:* 221-225. PubMed doi:10.1055/s-2007-1022263

[28] *Wiegerinck MAHM, Portmann J, Donker TH, Thijssen JHH.* In vivo uptake and subcellular distribution of tritium labelled estrogens in human endometrium, myometrium, and vagina. J Clin Endocrinol Metab. 1983; *19 (1C):* 939-945.

[29] *Hahn RG.* Compliance considerations with estrogen replacement: withdrawal bleeding and other factors. Am J Obstet Gynecol. 1989; *161:* 1854-1858. PubMed

[30] *Stovall DW, Pinkerton JV.* Estrogen agonists/antagonists in combination with estrogen for prevention and treatment of menopause-associated signs and symptoms. Womens Health (Lond Engl). 2008; *4:* 257-268. doi:10.2217/17455057.4.3.257 PubMed