Gestodene and desogestrel do not have a different influence on concentration profiles of ethinylestradiol in women taking oral contraceptives – results of isotope dilution mass spectrometry measurements

Lothar Siekmann, Anita Siekmann, Frank Bidlingmaier, Klaus Brill¹ and Manfred Albring¹

Institut für Klinische Biochemie, Universität Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany and ¹Schering AG, Max-Dohrn-Str. 10, D-10589 Berlin, Germany

(Correspondence should be addressed to L Siekmann)

Abstract

Objectives: A new method for the quantitative determination of 17 α -ethinylestradiol-17 β (EE₂) in serum is presented here based on the principle of isotope dilution mass spectrometry (IDMS) with [¹³C]EE₂ as internal standard. The technique was used to determine the concentration profiles of EE₂ in the serum of female subjects who had taken oral contraceptives with different progestin components. The method has proved to be very reliable with respect to trueness, specificity, precision and detection sensitivity and offers considerable advantages compared with the immunological methods of measurement used to date.

Study design: Forty-seven female volunteers took two different oral contraceptives containing EE₂ combined with different progestins in accordance with a cross-over design. After the administration of 30 μ g EE₂ combined with 75 μ g gestodene (EE₂/GSD) or 150 μ g desogestrel (EE₂/DES), blood samples were taken from the subjects on certain days and in certain previously specified cycles in the course of 12 h after medication.

Results and conclusions: The biometric analysis of the results showed that the concentration profiles of EE₂ were, in their statistics, significantly equivalent after the administration of either of the two oral contraceptives. The sometimes contradictory results found in former studies after the administration of the different contraceptives were presumably due to the methodological shortcomings of the radio-immunological measurement technique. With the use of the highly accurate and specific technique of IDMS it can now be unequivocally established that the different progestins in the tested oral contraceptives have no influence on the bioavailability of EE₂ (area under EE₂ serum concentration curves, as usually defined in pharmacokinetics).

European Journal of Endocrinology 139 167–177

Introduction

17 α -Ethinylestradiol-17 β (EE₂) is contained as an estrogen component in all oral contraceptives used today. In low-dose preparations, 30 μ g EE₂ are combined with 75 μ g gestodene (EE₂/GSD) or with 150 μ g desogestrel (EE₂/DES) as progestins. Controversy has flared up in recent years as to whether the progestin influences the metabolism of the estrogens. In particular it has been claimed that gestodene delays the metabolism of EE₂ and thus leads to serum levels which are, on average, higher by 70% than those during treatment with desogestrel (1). Other well-documented clinical studies have shown, however, that, whichever progestin is used, there is no difference in the bioavailability of EE₂ (2–7). The controversial results have recently been discussed and reviewed (8).

The calculation of the bioavailability in these investigations is based on the measurement of the

areas under the curves (AUC) of serum EE₂ concentrations, which were determined in volunteer trial subjects after administration of the preparations. In all of the studies, radioimmunological measurement procedures were used to determine EE₂ in the serum. It is well known, however, that immunological procedures are not very reliable, especially when, as in the present case, the concentrations to be measured in the analytical sample are very low. This is also apparent, *inter alia*, from the characteristics described for the immunological methods of determining EE₂. A high specificity of the test procedure can be assumed when a very slight cross-reactivity of the antibody used against a number of sex steroids is found, but this is contradicted by blank measurements of EE₂ during the dose-free interval (6). Fluctuating EE₂ blank results of up to 25 pg/ml were measured, depending on the day of the cycle. These high and fluctuating EE₂ blanks are presumably caused by as yet unknown substances from the serum matrix which

simulate EE₂ during the immunological determination. This suggests that the radioimmunological test methods used probably do not provide very good accuracy and specificity. To date there have been no reports on comparative investigations using reference methods which make it possible to draw objective conclusions on the accuracy of the immunoassays used. In view of the discrepant findings in the bioavailability studies available, the question arises whether or not the value of such pharmacokinetic studies may be limited to a considerable extent, or they may even be faulty because of the unreliability of the immunological EE₂ determinations.

In the study described here, therefore, a completely different principle of analysis, based on earlier investigations (9), was used to determine the serum EE₂; this method – isotope dilution mass spectrometry (IDMS) – is characterized by improved specificity, accuracy and precision compared with radioimmunoassay. The principle of the method was first described in 1970 (10) and it is now recognized as one of the most reliable analytical procedures in biochemical and clinical chemical analysis.

In the pharmacokinetic investigations described here, analysis by IDMS was used to determine EE₂ in 47 female subjects after the administration of EE₂/DES and EE₂/GSD.

The purpose of the investigation was to determine whether progestins (gestodene and desogestrel) in oral contraceptives influence the pharmacokinetics of nifedipine, which was administered to the subjects simultaneously. In this connection it was of interest to find out whether the bioavailability of EE₂ was equivalent after the administration of the two contraceptives. This report deals exclusively with the methodological aspects of EE₂ determination by mass spectrometry and the biometric results concerning the bioavailability of EE₂ after the administration of the different oral contraceptives. The biometric findings for the nifedipine kinetics will be reported elsewhere.

Materials and methods

Analysis of EE₂ in human serum by IDMS

Principle of the method A measured sample of serum is mixed with a defined amount of isotope-labeled EE₂

([¹³C]EE₂). The molecular structures of the labeled and the non-labeled EE₂ are shown in Fig. 1. After equilibration of the labeled steroid, added exogenously, with the endogenous steroids in the serum sample, purification of the steroid fraction is carried out through extraction and column chromatography. The steroids contained in the EE₂ fraction are converted into a derivative suitable for combined gas chromatography/mass spectrometry (GCMS). The derivatives are injected into a gas chromatography column, the end of which is connected to a mass spectrometer. During the GCMS analysis a characteristic ion is continuously recorded from the spectrum of the non-labeled EE₂ derivative while the corresponding ion is recorded simultaneously from the spectrum of the isotope-labeled EE₂ derivative. The results are calculated from the isotope ratios determined by mass spectrometry in samples and standard mixtures of labeled and non-labeled EE₂.

Measurement procedure Samples of 1.3 ml serum taken from female subjects during the medication cycles were each mixed with 25 µl of an alcoholic solution which contained 82 pg [¹³C]EE₂. A 1 + 9 diluted solution of the isotope-labeled steroid (8.2 pg [¹³C]EE₂/25 µl) was used to analyze the EE₂ in the serum samples taken from volunteers during the medication-free cycles (pre-period and washout cycles). The samples were shaken gently at room temperature for 30 min to equilibrate the labeled and non-labeled steroids. The steroids were then extracted with 15 ml dichloromethane (Merck, Darmstadt, Germany) by vigorous shaking. The aqueous phase was siphoned off and the remaining organic phase was washed twice with 1 ml distilled water in each case. The extract was mixed with 20 µl of an alcoholic solution which contained 2 µg α-naphthol (as carrier substance). The organic solvent was evaporated to dryness under nitrogen and the dry residue was dissolved twice with 250 µl of a mixture of dichloromethane, cyclohexane, methanol and water (100:80:15:1 v/v); this was then transferred to a chromatography column containing 1 g Sephadex-LH20 (Pharmacia, Freiburg, Germany). Chromatography was carried out with the same solvent mixture; 9.25 ml of column eluate were discarded and the EE₂ fraction (1.5 ml) was then collected in conical reaction vials which contained 2 µg α-naphthol. The precise retention volume of EE₂ during Sephadex

Figure 1 Molecular structure of EE₂ and [3,4-¹³C₂]EE₂.

chromatography must be determined from time to time using radioactive EE₂. The developing solvent was evaporated to dryness under nitrogen and the residue was reacted with a mixture (30 µl) of heptafluorobutyric anhydride and dry acetonitrile (1:4) for conversion into the heptafluorobutyric ester derivative. After a reaction time of 60 min at room temperature, the reagents were evaporated off in a vacuum centrifuge and dissolved in 10 µl dry cyclohexane for the GCMS analysis.

For calibration, ten standards were prepared from a primary standard solution in each analytical series: four standards contained 80 pg, three standards 40 pg, and three others 120 pg EE₂ each. The standards were pipetted into conical reaction vials, containing the same amount of isotope-labeled EE₂ as had been added to the serum sample (82 pg). EE₂ (Sigma Chem. Co., Deisenhofen, Germany) (reference standard), which was dried in a vacuum before being weighed, was used as non-labeled standard substance. The standards were converted to derivatives and dissolved under the same conditions as the analytical samples, but not subjected to column chromatography. In order to check for possible influence on quantities arising from the sample preparation procedure a duplicate of the 80 pg standard was carried through the whole analytical procedure (extraction, chromatography, derivative formation) in each analytical series. The resulting isotope ratios did not differ by more than 1.5% from those of the non-processed 80 pg standards.

A moving-needle solid injection system (11) was used for the injection of the samples and standards into the capillary gas chromatography column. The gas chromatography was carried out on an SE-52 column (30 m × 0.25 mm, film thickness 0.1 µm, temperature program: start at 210 °C, temperature program 30 °C/min to 240 °C, 5 °C/min to 260 °C, 40 °C/min to 310 °C, 2 min at 310 °C), carrier gas: helium at 80 kPa. Transfer line to mass spectrometer: deactivated fused silica capillary tube, internal diameter 0.15 mm.

For the mass-specific detection, the quadrupole mass spectrometer (MD-800, Fisons Instruments, Mainz, Germany) connected to the gas chromatograph was adjusted to the m/z values 474 and 476 (dwell time 50 ms respectively). The temperature of the ion source was 250 °C; the ionization energy was adjusted to 70 eV. The mass resolution of the instrument was focused to about 500 (10%-valley-definition). An integrated computer system was used to control the GCMS system, to record the mass-specific chromatograms, to integrate the peak areas, to determine the peak heights and to calculate the isotope ratios.

To calculate the analysis results from the isotope ratios in the calibrator mixtures and samples, we employed a mathematical procedure which has been reported in detail (12).

Analyses were carried out in 43 batches of six to nine samples (each in duplicate). For each batch a new set of standard mixtures was prepared. For quality control a

male donors' serum pool spiked with 51.2 pg/ml EE₂ was analyzed within each batch. As a limit for accepting the measurement results of a batch the maximum allowable deviation from the target value of the control sample was set to ± 3%. The 329 serum samples were analyzed in random order. The laboratory staff were not informed of the sample codes referring to the administration of either EE2/DES or EE2/GSD.

Synthesis of [¹³C]EE₂ A procedure based on a previously described method (13) was used for the synthesis of [3,4-¹³C₂]EE₂ from 2.4 mg [3,4-¹³C₂]estrone (Cambridge Isotope Laboratories, Woburn, MA, USA) and 16 mg lithium acetylide in 2.5 ml dimethylsulfoxide. After thin-layer chromatographic purification of the crude product, 0.5 mg (21%) [3,4-¹³C₂]EE₂ was obtained. The mass spectrometry analysis of this material resulted in a peak area ratio of <0.045 when the m/z values 474 and 476 were recorded.

Trial design for comparing EE₂ bioavailability after the administration of EE2/GSD and EE2/DES

Volunteers A total of 74 healthy female volunteers participated in this study. Study acceptance criteria specified that the Broca index of these women should not exceed ± 10% and that the women should not have used oral contraceptives for at least 3 months prior to the beginning of the study. After being fully informed through written material of the purpose of the trial, each participant gave written consent. Applicants were subjected to general, gynecological and laboratory examinations including an ultrasonic examination of the liver before being selected for participation in the study. Any contraindications for oral contraceptives were regarded as exclusion criteria. All general physical examinations at the beginning of the study as well as at later time points included the following: blood samples for evaluation of nifedipine kinetics, determination of body weight and blood pressure, a standard blood glucose test, determination of the white blood cell count, a pregnancy test and a systemic drug screening. The study was conducted according to the EC guidelines of Good Clinical Practices as well as the revised declaration of Helsinki. The study was reviewed and approved by an external ethics committee (Ethikkommission der Ärztekammer Hessen, Frankfurt a. M., Germany).

Study design The study was conducted as an open intraindividual comparison following ingestion of the two preparations for 3 months each. One formulation contained 75 µg gestodene in combination with 30 µg EE₂ (EE2/GSD) (Femovan, Schering AG, Berlin, Germany), while the second formulation contained 150 µg desogestrel in combination with 30 µg EE₂ (EE2/DES) (Marvelon, Organon, Oss, The Netherlands). A washout phase was

Table 1 Experimental design for the comparison of the bioavailability of EE₂ after the administration of EE2/GSD and EE2/DES.

Cycle	Day	Period	Cross-over design
1	7–12	Pre-period	Blank cycle
1	14–21	Pre-period	Blank cycle
2	14–21	Medication 1	1st treatment cycle
3	–	Medication 1	
4	14–21	Medication 1	3rd treatment cycle
5	–	Washout	
6	14–21	Washout	Blank cycle
7	14–21	Medication 2	1st treatment cycle
8	–	Medication 2	
9	14–21	Medication 2	3rd treatment cycle

scheduled between the two phases of administration. The two treatment sequences were as follows (see also Table 1):

(A) EE2/GSD for a period of three cycles, washout phase for two cycles and then EE2/DES from the sixth to the eighth cycle.

(B) EE2/DES for a period of three cycles, washout phase for two cycles and then EE2/GSD from the sixth to the eighth cycle.

In order to avoid major intra-individual differences, 71 volunteers were screened in the pretreatment cycle on days 7–17 and 14–21 for nifedipine kinetics. Only volunteers with a <20% variation in their intra-individual AUC_{0–12} and k_e (rate constant of elimination) were included in the study. Forty-seven women fulfilling the criteria were allocated at random to one of the two treatment sequences. Twenty milligrams nifedipine in non-sustained release form (Adalat, Bayer AG, Leverkusen, Germany) were administered in addition to the oral contraceptives on days 14–21 of the first and third cycles (phase A), the seventh and ninth cycles (phase B) and in the second cycle of the washout phase. Blood samples were collected via a venous outlet at 0, 20, 40, 60, 90, 120 and 150 min, as well as 3, 4, 6, 8 and 12 h following administration on one day between the 7th and 12th and the 14th and 21st day of the pretreatment cycle 1. Further samples were obtained on one day between the 14th and 21st day of cycle 2 and 4 (medication 1), cycle 5 (washout period) and cycle 7 and 9 (medication 2) as shown in Table 1.

Four and 10 h after ingestion of the medication a standardized lunch and a second meal were given.

The statistical hypotheses to be tested by the studies are described as follows:

$$H_0 : \frac{AUC_{12}(EE2/GSD)}{AUC_{12}(EE2/DES)} \leq 0.8$$

$$\frac{AUC_{12}(EE2/GSD)}{AUC_{12}(EE2/DES)} \geq 1.2$$

$$H_1 : 0.8 \leq \frac{AUC_{12}(EE2/GSD)}{AUC_{12}(EE2/DES)} \leq 1.2$$

The bioavailabilities of EE₂ after administration of EE2/GSD and EE2/DES are accepted as equivalent when they do not differ by more than 20%.

Preparation of weighted serum pools To determine bioavailability, the serum concentrations of the substance to be investigated are usually monitored for a certain period of time after the preparation has been taken (e.g. after 4, 12 or 24 h). The AUC of the serum concentration, calculated by the trapezoidal rule, represents the measure of bioavailability. This procedure necessitates a large number of analyses, since each blood sample must be measured separately.

Recently a new procedure has been presented (14) whereby only one measurement has to be made on each investigation day to determine bioavailability: a weighted serum pool is prepared from the individual serum samples. The concentration determined in the pool multiplied by the observation period (12 h) represents a measure of the AUC_{0–12}. The individual volumes to be taken for the pool are all the greater, the longer the interval of time is before and after the blood sampling in question. Table 2 lists the individual volumes of the blood samples used to form the serum pools for the EE₂ determinations; these were based on the sampling times and a target volume of 3 ml. In cases where individual blood samples were missing ($n=6$ of 4277), larger volumes were used from the blood samples taken before and after the missing sample to correspond with the longer interval of time.

A double determination was carried out from each 'weighted serum pool' using 2×1.3 ml in each case. In some cases ($n=43$ of 329) the amount of serum was not sufficient for the preparation of a 3 ml pool; then the individual volumes for the preparation of the pool were halved. Out of the resulting pool of 1.5 ml, a double determination was carried out with 0.65 ml from each subject during the medication cycle, and single

Table 2 Production of 'weighted serum pools' for the determination of the AUC_{0–12}

Time after administration (min)	Calculation of the volume	Volume (ml)
0	$1/6 \times 1/12 \times 3 = 1/24$	0.042
20	$1/3 \times 1/12 \times 3 = 1/12$	0.083
40	$1/3 \times 1/12 \times 3 = 1/12$	0.083
60	$5/12 \times 1/12 \times 3 = 5/48$	0.104
90	$1/2 \times 1/12 \times 3 = 3/24$	0.125
120	$1/2 \times 1/12 \times 3 = 3/24$	0.125
150	$1/2 \times 3/12 \times 3 = 3/24$	0.125
180	$3/4 \times 1/12 \times 3 = 9/48$	0.188
240	$1 \times 1/12 \times 3 = 3/12$	0.250
300	$1 \times 1/12 \times 3 = 3/12$	0.250
360	$3/2 \times 1/12 \times 3 = 9/24$	0.375
480	$3 \times 1/12 \times 3 = 9/12$	0.750
720	$2 \times 1/12 \times 3 = 6/12$	0.500
		Total = 3.000

determinations with 1.3 ml during the medication-free pre-period and the washout cycles.

In the case of a few pools ($n = 3$ of 329), the 12 h sample was missing or the amount of serum was too small; in these cases the samples from 0 to 480 min were pooled and in addition a determination was carried out on the individual samples taken at 300, 360 and 480 min, enabling us to form an estimate on the total pool of 720 min.

To ensure the greatest possible precision and accuracy in dosing the weighted individual volumes, adjustable piston pipettes operating with disposable tips and air cushions were calibrated gravimetrically and validated by repeated dispensing.

Results

Characteristics of the analytical procedure

Precision of the method The precision of the IDMS analysis of EE₂ in serum was ascertained through double determinations carried out on 43 days on a male donors' serum pool to which EE₂ had been added (51.2 pg/ml). The mean value was 51.7 pg/ml. The standard deviation (s.d.) calculated from the double determinations (15), which represents a measure for the 'within series' precision, was 0.64 pg/ml (=1.2%). The s.d. from the 43 measurement results, as a measure of the 'series to series' precision, was calculated to be 0.80 pg/ml (=1.5%).

Reproducibility of the procedure was further tested by including control samples of male donors' serum pools spiked with various amounts of EE₂ in four to eight analytical series. The relative s.d.s for the control pools are summarized in Table 3. The concentrations were in the range from 19 to 127 pg/ml thereby covering the concentration range of 99% of the serum samples from the treatment cycles.

The double determinations ($n = 188$) of EE₂ carried out on the pools of the female subjects during the medication periods resulted in an s.d. of 0.81 pg/ml. The mean concentration was 64.4 pg/ml. The relative s.d. calculated from the double determinations was 1.3%. This result confirms the 'within series' precision from

double determinations on a male donors' pool to which 51.2 pg/ml EE₂ had been added.

Lower detection limit Blank value measurements on a serum pool from male donors carried out on ten different days resulted in a mean measurement value of 0.390 pg/ml (s.d. 0.145 pg/ml). Taking into account the fact that double determinations were carried out on the subjects' sera, the following was obtained as the lower detection limit:

$$x = x(\min) * \frac{3 * S.D.}{\sqrt{2}} = 0.699 \text{ pg/ml}$$

Although the samples from the premedication and washout cycles do not represent real blanks it should be noted here that their results are of similar magnitude; the double determinations ($n = 120$) of EE₂ carried out on these pools resulted in a mean concentration of 0.80 pg/ml (s.d. 0.33 pg/ml).

Lower limit of quantitation This was estimated from the data obtained in the reproducibility experiments (Table 3). It is reasonable to assume that the lower limit of quantitation (relative s.d. not exceeding 20%) is approximately 3 pg/ml.

Upper detection limit A reduced precision of the analytical results must be expected when the serum sample contains more than 160 pg EE₂ and only 82 pg internal standard. In this case the analysis should be repeated with a correspondingly smaller amount of serum. Otherwise there are no restrictions on the upper measurement range.

Recovery The recovery was calculated from the five serum pools to which defined amounts of EE₂ had been added and which were also used for testing reproducibility. Table 3 shows the concentrations measured in different analytical series compared with the target concentrations. In the five different concentration ranges between 19 and 127 pg/ml, the relative recoveries were between 101.0 and 104.2%.

Bioavailability of EE₂ after the administration of EE₂/GSD and EE₂/DES

The EE₂ concentrations in 188 weighted serum pools obtained from female subjects during the medication cycles and in 141 pools from the pre-periods and washout cycles were determined through a double determination by means of IDMS analysis. The laboratory measuring the EE₂ did not know whether the samples came from the EE₂/GSD or EE₂/DES medication cycles. The results of 1 of the 47 female subjects had to be excluded because the EE₂ measurements showed that on one day she had apparently not taken the EE₂/GSD preparation at the planned time.

Table 3 Reproducibility of EE₂ measurements determined after its addition to a male serum pool in different analytical series.

Added EE ₂ (pg/ml)	Found EE ₂ (pg/ml)	<i>n</i>	Relative s.d. (%)	Recovery of EE ₂ (%)
18.63	19.40	5	2.2	104.2
38.03	38.52	5	1.9	101.3
51.20	51.70	43	1.5	101.0
58.33	59.06	4	0.8	101.3
75.89	77.50	6	1.1	102.1
125.94	127.34	8	1.2	101.1

Table 4 Characteristic data for the EE₂ AUC₀₋₁₂ (pg×h/ml) after the administration of EE2/GSD and EE2/DES.

Sequence	Cycle	Medication	Minimum	Maximum	Median	Mean value	s.d.
EE2/GSD–EE2/DES (<i>n</i> = 20)	1		3.7	242.7	10.0	23.5	52.2
	2	EE2/GSD	342.5	1304.2	774.9	755.0	237.9
	4	EE2/GSD	105.2	1325.8	819.7	771.5	303.4
	6		2.3	19.9	8.9	9.2	5.1
	7	EE2/DES	173.0	1380.2	809.7	805.0	294.7
	9	EE2/DES	101.9	1430.9	857.7	796.9	305.2
EE2/DES–EE2/GSD (<i>n</i> = 26)	1		2.4	28.0	7.5	8.8	5.4
	2	EE2/DES	256.9	1344.2	662.9	728.3	263.1
	4	EE2/DES	243.6	1431.7	763.4	840.7	293.4
	6		0.8	39.4	6.6	9.3	8.9
	7	EE2/GSD	364.1	1400.5	738.1	770.9	270.9
	9	EE2/GSD	108.9	1428.4	736.5	774.2	273.4

The results of the cross-over study are shown in Table 4. Figure 2 shows the results of the female subjects (*n* = 20) who took EE2/GSD first (second to fourth cycle) followed by EE2/DES (seventh to ninth cycle) and of those (*n* = 26) who took the medication in the reverse sequence.

If one looks at the results of the blank cycles (first and sixth cycle), no statistically significant differences can be seen between the AUCs before EE2/GSD and before EE2/DES. There is a tendency towards a higher value in cycle 1 compared with cycle 2, which may be caused by the obviously outlying maximum value (242.7 pg×h/ml). But this would probably not affect the overall result of considerably higher EE₂ levels after EE2/GSD and EE2/DES.

Table 4 and Fig. 2 show that the sequence of the medication causes no significant difference in the AUCs

during the treatment periods 2, 4, 7 and 9. It is therefore permissible, for the purpose of descriptive statistics, to combine the results of the treatment periods in which the contraceptive formulas were taken in the sequence EE2/GSD–EE2/DES with the treatment periods where the contraceptives were taken in the reverse order. This is shown in Table 5.

The changes seen in the third compared with the first treatment cycle (cycle 4 versus 2 and cycle 9 versus 7) are not statistically significant (EE2/GSD: *P* = 0.658; EE2/DES: *P* = 0.406).

Figure 3 shows the AUCs determined for each female subject taking EE2/GSD compared with the AUCs of those taking EE2/DES (mean values from the first and third treatment cycle in each case). The equivalence of the bioavailability, already recognizable from the graphs, was confirmed by a statistical equivalence test,

Figure 2 Mean values and s.d.s of the EE2 AUC₀₋₁₂ during the medication periods and during the medication-free pre-periods and washout cycles. The female subjects (*n* = 20) who received the oral contraceptives in the first and second treatment periods in the sequence EE2/GSD–EE2/DES and the group of subjects (*n* = 26) who received EE2/DES first and then EE2/GSD are shown separately.

Table 5 Characteristic data for the EE₂ AUC₀₋₁₂ (pg × h/ml) after the administration of EE2/GSD and EE2/DES using the combined results from different treatment sequences.

Cycle	EE2/GSD		EE2/DES	
	Mean	S.D.	Mean	S.D.
1. Treatment cycle (cycle 2 and 7)	764.0	254.4	761.6	276.8
2. Treatment cycle (cycle 4 and 9)	773.0	283.5	821.7	296.0

which was carried out using the confidence interval inclusion method with an equivalence range of 0.8–1.25 in the multiplicative model:

Equivalence test ($n = 46$)

Treatment cycle 1 (cycles 2 and 7): $P = 0.0031$

Treatment cycle 3 (cycles 4 and 9): $P = 0.0193$

Global (mean value from all treatment cycles): $P < 0.0001$

Table 6 shows the relative bioavailability of EE₂ after administration of EE2/GSD versus EE2/DES, as well as the 90% confidence intervals. In the first and third treatment cycle, the relative bioavailability is 101.8 and 93.3%. The global relative bioavailability of EE₂ (after administration of EE2/GSD versus EE2/DES) for all treatment cycles is 97.5% with a 90% confidence interval between 89.5 and 106.0%.

Discussion

The primary objective of this investigation was to develop a method for determining EE₂ in human

Table 6 Relative bioavailability of EE₂ after administration of EE2/GSD versus EE2/DES.

Treatment cycle	Bioavailability (%)	90% confidence interval (%)	P value*
1	101.8	90.2–115.0	0.0031
3	93.3	82.6–105.3	0.0193
Global	97.5	89.6–106.0	0.0001

*Test of equivalence with the equivalence range 80–125%.

serum that would be distinctly more reliable than the immunological procedures described to date. By using radioimmunoassay procedures one clinical study (1) produced results which were contradictory to several other investigations (2–7) regarding the pharmacokinetics of EE₂ after the combination of this estrogen with different progestins. Methodological factors must presumably be considered as the possible cause of contradictory results from different studies. A possible source for the discrepant results of the first study may be a systematic error as discussed recently (8); the authors of the study (1) did not analyze the samples from the two groups (EE2/DES and EE2/GSD) in random order but at different occasions in separate batches for each group. The radioimmunoassay applied in this study possibly had insufficient stability to furnish reproducible results from day to day and from batch to batch. Therefore, the difference observed by the authors of the study (1) may have been caused by the fact that the samples were not analyzed in random order and the immunoassay reproducibility was poor.

It is generally known that radioimmunological procedures are not particularly reliable, especially

Figure 3 Presentation of the EE₂ AUC₀₋₁₂ with the use of EE2/GSD versus the EE₂ AUC₀₋₁₂ with the use of EE2/DES (mean values from the first and third treatment cycle).

when dealing with concentration determinations in the femtomole range.

There is no question that the validity of pharmacokinetic equivalence investigations depends to a large extent upon the reliability of the analytical procedure used. In the field of clinical chemical laboratory diagnosis and in clinical endocrinology, the technique of IDMS is today regarded as the method of choice for developing reference measurement procedures (12, 16–18). The logical assumption therefore is that, despite the high cost in terms of personnel and equipment, this analytical principle should also be used for pharmacokinetic investigations, especially if there are doubts about the reliability of alternative methods.

The accuracy of the IDMS method used here results from combining two analytical procedures: capillary gas chromatography and mass spectrometry, each of which possesses a high specificity in itself. The mass spectrometer is adjusted to the ions characteristic for the substance to be investigated thus providing a detection system whose specificity can be adapted to the measurement problem in question. In the present instance the mass spectrometer was set to the ions m/z 474 and 476, characteristic for the derivative of EE₂ and to the corresponding isotope-labeled reference compound. Thus, only those components that form the specified ions in the mass spectrometer are selectively indicated during the gas chromatographic analysis. Figure 4a and b show the mass-specific detection of EE₂ and [¹³C]EE₂ after processing a serum sample. Although the analytical sample still contains hundreds of impurities from the biological material (even after extraction and separation by column chromatography), only very few are indicated during the gas chromatography because of the mass-specific detection. EE₂ can be unequivocally identified amongst these impurities by means of the characteristic gas chromatographic retention time. Not only is the specificity of the detection of great importance for analytical accuracy but also the precise control of the preparation losses during the analytical procedure. This analytical method gives optimum control of the recovery due to the use of the isotope dilution principle.

Radioimmunological measurement procedures for the determination of EE₂ have been evaluated using a GCMS method (19). Although the procedure is not described in detail in that paper, it is obvious that this is not an isotope dilution analysis. In view of the absence of recovery control, it is not surprising, therefore, that the 10% coefficient of variation of the GCMS method and the lower detection limit of 10 pg/ml are hardly an improvement on the use of immunological test procedures. The fact that, in comparative investigations, the EE₂ concentrations found in the relevant range up to 150 pg/ml were always higher than the radioimmunological measurements is not exactly an argument in favor of the reliability of the GCMS method used. It is questionable whether a simple GCMS method without

the use of an isotope-labeled internal standard is suitable for the evaluation of immunological measurement techniques.

The descriptions of the immunological methods used to date for determining EE₂ hardly provide useful information on accuracy. Statements made about accuracy are, in fact, often about the results of recovery experiments. To date no comparative analyses are available with reference methods that could provide data on the accuracy of the immunological EE₂ determination procedure. The high blank values of up to 25 pg/ml determined in the medication-free intervals and reported in all the relevant publications suggest, however, that the accuracy of the immunological tests is not very high. In the case of these so-called 'blanks' one is probably dealing here with the effect of interfering substances which simulate EE₂ because of the lack of specificity of the antigen/antibody reaction. It has been shown that the blank values found can be extremely variable (6). These authors quite rightly ask whether the serum blank values should be subtracted from the measurement values as was done in previous investigations (1). It is conceivable, however, that the concentration of interfering substances is altered by the influence of oral contraceptives.

The lower detection limits for the immunological determination procedures used to date are between 8 pg/ml (5) and 25 pg/ml (6). A mean serum concentration of 64 pg/ml means that a large proportion of the measurements was in fact carried out only just above the lower detection limit. With the mass spectrometry procedure used in our study, a lower detection limit of only 0.7 pg/ml was determined with the use of a pool from male donors. From determinations for the female subjects during the pre-periods and washout cycles, the detection limit was calculated at 1.5 pg/ml. This considerably improved lower detection limit, compared with immunological methods, is an important prerequisite for the reliability of the IDMS method.

The question, however, arises, why different blank values were found in the serum pool of men (0.39 pg/ml) and in the medication-free and washout sera of women (0.8 pg/ml). The cause for the blanks in the IDMS method could, on the one hand, be electronic noise dependent on the technical performance of the instrument and, on the other, 'chemical' noise. And indeed it was found that in the 'blank value samples' spiked with only 8.2 pg [¹³C]EE₂, other additional substances with comparable intensity become visible, which produce signals in the selected ion chromatograms at the masses 474 and 476. These differ, however, in their gas chromatographic retention time from the derivatives of the EE₂ and the ¹³C-labeled compound. However, it cannot be completely ruled out that one or more substances exhibit the same retention time as EE₂ and therefore interfere with its detection. Possibly there are higher concentrations of such interfering substances in the serum of women than in that of men, which leads to

(a)

Sample ID: 8/ pool

ASI16 Sm (SG, 2x2)

(b)

Sample ID: 8/ pool

ASI16 Sm (SG, 2x3)

Figure 4 (a) Isotope dilution mass spectrometry analysis of the heptafluorobutyric ester derivative of EE₂ after processing a serum sample. Upper chromatogram, recording of [^{13}C]EE₂ at m/z 476; lower chromatogram, recording of the non-labeled EE₂ at m/z 474. (b) Enlarged section of the EE₂ peak from the mass-specific chromatograms shown in (a). SIR, selected ion recordings.

Figure 5 EE₂ serum concentration curve after the oral administration of 30 µg EE₂ and 75 µg gestodene (EE₂/GSD).

an increase in the lower limit of detection. The blank measurements and the phenomenon of different blanks for men and women make the limits of this methodology very clear. With reference to the evaluation of bioavailability within the framework of the present study, however, this phenomenon of blank values of about 0.8 pg/ml is quantitatively insignificant.

The precision calculated from repeated 'series to series' measurements is a parameter of reliability reported in all of the available studies that use an immunological measurement technique. Thus a coefficient of variation from 'series to series' of 15.1% (with a mean concentration of 70 pg/ml) was reported (1). This roughly corresponds to the data of another paper reporting a 'series to series' coefficient of variation of 16.0% with a mean concentration of 48.7 pg/ml (6). With an EE₂ concentration of 143 pg/ml, the coefficient of variation lies at 9.4%. In a recent paper (7) 'series to series' coefficients of variation of 13.3% are reported at a concentration of 93.2 pg/ml and of 12.6% at 192.2 pg/ml. By comparison with the immunological procedures, the IDMS method described here has proved to be very precise. The coefficient of variation was generally about 1.2% 'within series' and approximately 1.5% 'series to series'.

It is reported (14) that, with the weighted pools method used here, the variance of the bioavailability

determination increases in a ratio of $2.625 \times s^2$ to $18.625 \times s^2$, i.e. by a factor of 7.1 compared with the trapezoidal method. If, however, one looks at the scatters of s^2 , which are on an order of magnitude of 100 with immunological methods compared with 0.97 with IDMS, then the contributions to the individual variances are: $2.652 \times 100 = 265$ for the trapezoidal method with immunological procedures and $18.625 \times 0.97 = 18.1$ for the 'weighted pools' method using mass spectrometry.

This results in a distinct advantage for the use of IDMS determination, even though the preconditions are less good through the weighted pools method. In order to compare the 'weighted pools' method with the usual 'trapezoidal method' from EE₂ serum concentration curves, individual measurements in the course of 12 h and also a 'weighted pool' of the 13 individual samples were carried out for the 1 day measurement of four subjects. Figure 5 shows a typical EE₂ serum concentration curve, such as was obtained with the use of the IDMS analysis for one female subject. Table 7 shows the results of the 1 day measurements of four subjects as a comparison. There is a good correlation between the two methods, so that no reservations should exist as to the use of 'weighted pools', as already reported (14).

Taken as a whole, the IDMS analysis of the serum EE₂

Table 7 Comparison of the EE₂ AUC₀₋₁₂ (pg × h/ml) determined from 13 individual measurements by the 'trapezoidal method' and from a concentration measurement in the 'weighted serum pool'.

Subject	Weighted pool AUC ₀₋₁₂	Trapezoidal method AUC ₀₋₁₂	Difference in AUC ₀₋₁₂	Relative difference (%)
011	992.2	1008.5	-16.3	-1.6
249	1030.2	1059.7	-29.5	-2.8
265	990.6	959.4	31.2	+3.3
270	1154.0	1164.7	-10.7	-0.9

described here must be seen as a quantitative analytical procedure which is distinctly superior to the immunological methods in specificity, trueness, precision and lower limit of quantitation. The use of the method described here for determining EE₂ in a cross-over study that administered nifedipine as well as EE₂ and the different progestins did not afford any evidence that the bioavailability of EE₂ differs at all after the administration of either EE₂/GSD or EE₂/DES. The possibility that the progestins (gestodene and desogestrel) might have differing effects on the metabolism of EE₂ can therefore be excluded.

Acknowledgements

The authors would like to thank Dr H J Wildgrube, Institute for Applied Diagnostics, D-63450 Hanau for collecting the blood samples for EE₂ analyses. We also wish to thank Mrs Anja Schumacher for the careful synthesis of the [¹³C]EE₂.

References

- Jung-Hoffmann C & Kuhl H. Interaction with the pharmacokinetics of ethinylestradiol and progestogens in oral contraceptives. *Contraception* 1989 **40** 299–312.
- Hümpel W, Täuber U, Kuhn W, Pfeffer M, Brill K, Heithecker R *et al.* Comparison of serum ethinyl estradiol, sex-hormone-binding globulin, corticoid-binding globulin and cortisol levels in women using two low-dose combined oral contraceptives. *Hormone Research* 1990 **33** 35–39.
- Kuhn W, Hümpel M, Schütt B, Louton T, Steinberg B & Gansau C. Relative bioavailability of ethinyl estradiol from two different oral contraceptive formulations after single oral administration to 18 women in an intraindividual cross-over design. *Hormone Research* 1990 **33** 40–44.
- Kuhn W, Back D, Power J, Schütt B & Louton T. Concentration of ethinyl estradiol in the serum of 31 young women following a treatment period of three months with two low-dose oral contraceptives in an intraindividual cross-over design. *Hormone Research* 1991 **36** 63–69.
- Orme M, Back DJ, Ward S & Green S. The pharmacokinetics of ethinylestradiol in the presence and absence of gestodene and desogestrel. *Contraception* 1991 **43** 305–316.
- Dibbelt L, Knuppen R, Jütting G, Heimann S, Klipping CO & Parikka-Olexik H. Group comparison of serum ethinyl estradiol, SHBG and CBG levels in 83 women using two low-dose combination oral contraceptives for three months. *Contraception* 1991 **43** 1–21.
- Hammerstein J, Daume E, Simon A, Winkler HU, Schindler AE, Back DJ *et al.* Influence of gestodene and desogestrel as components of low-dose oral contraceptives on the pharmacokinetics of ethinyl estradiol (EE₂), on serum CBG and on urinary cortisol and 6 β -hydroxycortisol. *Contraception* 1993 **47** 263–281.
- Stanzcyk FZ. Pharmacokinetics of the new progestogens and influence of gestodene and desogestrel on ethinylestradiol metabolism. *Contraception* 1997 **55** 273–282.
- Siekman L, Siekman A & Breuer H. Measurement by isotope dilution mass spectrometry of 17 α -ethinyloestradiol-17 β and norethisterone in serum of women taking oral contraceptives. *Environmental and Biomedical Mass Spectrometry* 1980 **7** 511–514.
- Siekman L, Hoppen HO & Breuer H. Zur gaschromatographisch-massenspektrometrischen Bestimmung von Steroidhormonen in Körperflüssigkeiten unter Verwendung eines Multiple Ion Detectors (Fragmentographie). *Zeitschrift für Analytische Chemie* 1970 **252** 294–298.
- Van den Berg PM & Cox TPH. An all-glass solid sampling device for open tubular columns in gas chromatography. *Chromatographia* 1972 **5** 301–305.
- Siekman L & Breuer H. Determination of cortisol in human plasma by isotope dilution-mass spectrometry. *Journal of Clinical Chemistry and Clinical Biochemistry* 1982 **20** 883–892.
- Rao PN. Preparation of 9 α ,11 α -tritiated 17 α -ethinyloestradiol, mestranol, estradiol-17 β , and norethindrone. *Steroids* 1971 **18** 219–229.
- Louton T, Kuhn W, Dibbelt L & Knuppen R. Weighted serum pools in comparison to the trapezoidal rule for estimating AUCs for ethinyl estradiol. The relationship of the variance of the determination to the interindividual variance. *European Journal of Clinical Pharmacology* 1994 **46** 77–81.
- Snedecor GW. Query. *Biometrics* 1952 **8** 85–86.
- Siekman L, Spiegelhalter B & Breuer H. Bestimmung von Aldosteron im Plasma des Menschen unter Verwendung der Kombination Gaschromatographie/ Massenspektrometrie. *Zeitschrift für Analytische Chemie* 1972 **261** 377–381.
- Siekman L. Determination of steroid hormones by the use of isotope dilution-mass spectrometry: a definitive method in clinical chemistry. *Journal of Steroid Biochemistry* 1979 **11** 117–123.
- Siekman L. Determination of oestradiol-17 β in human plasma by isotope dilution-mass spectrometry. *Journal of Clinical Chemistry and Clinical Biochemistry* 1984 **22** 551–557.
- Kuhn W, Louton T, Back DJ & Michaelis K. Radioimmunological analysis of ethinylestradiol in human serum. Validation of the method and comparison with a gas chromatographic/mass spectrometric assay. *Arzneimittel Forschung* 1993 **43** 16–21.

Received 31 December 1997

Accepted 27 April 1998