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Bioequivalence of of Two Oral Contraceptive Drugs Containing Ethinylestradiol and Drospirenone in Healthy Female Volunteers

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Abstract - The bioavailability and bioequivalence of two different film coated tablets containing ethinylestradiol and drospirenone were investigated in 36 healthy female volunteers after oral single-dose administration. The study was performed according to a single-center, randomized, single-dose, 2-way cross-over design with a wash-out phase of 28 days. Blood samples for pharmacokinetic profiling were taken post-dose up to 72 h (ethinylestradiol) and 144 h (drospirenone). Ethinylestradiol and drospirenone plasma concentrations were determined with a validated LC-MS/MS method. Bioequivalence between the products was determined by calculating 90% confidence intervals (90% I.C) for the ratio of AUC_{0-t} and C_{max} values for the test and reference products, using logarithmic transformed data. The 90% confidence intervals of ethinylestradiol were 89.13% – 95.32%, and 88.13% – 96.38%, respectively. The 90% confidence intervals of drospirenone were 94.50% – 102.12%, and 95.11% – 111.11%, respectively. Since the 90% confidence intervals for C_{max} and AUC_{0-t} were within the 80 – 125% interval proposed by Food and Drug Administration, it was concluded that the two ethinylestradiol and drospirenone formulations are bioequivalent in their rate and extent of absorption.

I. INTRODUCTION

Combination contraceptives are most effective means for contraception excluding sterilization. Contraceptives are hormonal agents; combination oral contraceptives contain both an estrogen (ethinylestradiol or mestranol) and a progestogen (many different progestogens are utilized throughout the world). Endogenous estrogens are largely responsible for the development and maintenance of the female reproductive system and secondary sexual characteristics. Estrogens act through binding to nuclear receptors in estrogen-responsive tissues. These will vary in proportion from tissue to tissue. Circulating estrogens modulate the pituitary secretion of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), through a negative feedback mechanism. Drospirenone is a synthetic progestin and spironolactone analog with antimineralocorticoid activity. In animals and in vitro, drospirenone has antiandrogenic activity, but no glucocorticoid, antiglucocorticoid, estrogenic, or androgenic activity. Progestins counter estrogenic effects by decreasing the number of nuclear estradiol receptors and suppressing epithelial DNA synthesis in endometrial tissue. [1-5]

The primary estrogen used in oral contraceptives is ethinylestradiol. 17-Ethinylestradiol (EE), a synthetic estrogen developed in 1938, is an essential constituent of oral contraceptives, which have been widely prescribed since the 1970s. [6] In general, ethinylestradiol is used in combination to prevent pregnancy in women. [7,8] The mean bioavailability of EE is reported to be 45%. [9,10] Its metabolism occurs mainly in the liver and at least 10 metabolites of 17EE have been isolated from human urine, with the 2-hydroxy species being the major metabolites. [11,12]

Drospirenone is a novel synthetic progestogen with a pharmacological profile similar to that of natural progesterone. An analog of spironolactone, drospirenone has antimineralocorticoid and antiandrogenic activity. [13-17] It is almost completely metabolized: less than 1% of the administered dose is excreted in the urine as unchanged drug. The metabolites of drospirenone undergo both hepatic and renal elimination. Based on receptor-binding studies, the metabolites excreted in urine are devoid of pharmacologic activity. [18-20] The compound is part of certain birth control formulations. Combined with ethinyl estradiol in oral contraceptive formulations, drospirenone-containing contraceptives have similar efficacy and safety profiles to other low-dose oral contraceptives, but seem to offer improved tolerability with regard to weight gain, mood changes, acne and treatment of a severe form of the premenstrual syndrome called premenstrual dysphoric disorder . [21,22]

The aim of this study was to compare in healthy volunteers, the pharmacokinetics profiles and evaluate the bioequivalence of one test formulation containing 0.02 mg of ethinylestradiol and 3 mg of drospirenone, (test formulation). The test formulation was compared to one commercial formulation containing 0.02 mg of ethinylestradiol and 3 mg of drospirenone (reference formulation).

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II. Results

a) Demography and safety

Thirty one of the 36 enrolled subjects completed the study. Two subject dropped out after phase two for personal reasons. Three subjects dropped out before confinement for personal reasons and abnormal clinical laboratory investigations. Hence 31 completed cases for both treatments were available for analysis of ethinylestradiol and drospirenone plasma concentrations.The demographic characteristics of the study subjects are presented in **Table 1**, including age, height, weight and BMI. Ethinylestradiol and drospirenone were well tolerated at the administered dose. No serious adverse events occurred.

b) Pharmacokinetic and Statistical Analysis

The mean (± SD) plasma concentration-time profiles are presented in **Figure 01** (ethinylestradiol) and **Figure 02** (drospirenone) and the pharmacokinetic parameters of both substances are summarized in **Table 2** and **Table 3**.

The mean of Cmax of ethinylestradiol was 84.31 pg/mL in reference product and 77.76 pg/mL in test product. Both occurred 1.25 h after dose administration. Cmax of drospirenone was on average 56947.08 pg/mL in reference product and 58431.17 pg/mL in test product and occurred 1.25 h after dosing (reference) and 1 h (test). For ethinylestradiol, the geometric means of AUC0-∞, a measure of extent of absorption amount, were 854.86 pg.h/mL (reference) and 794.61 pg.h/mL (test). The geometric means of AUC0-∞ of drospirenone were 906099.00 pg.h/mL (reference) and 889520.52 pg.h/mL (test). The values of AUC0-t for ethinylestradiol were 807.17 pg.h/mL (reference) and 746.06 pg.h/mL (test). In the drospirenone evaluation the amounts of AUC0-t were 851151.59 pg.h/mL (reference) and 835564.88 pg.h/mL (test). No significant differences with respect to drug absorption were found. Elimination half-lives and elimination rate constants were well comparable between the different preparations.

The resulting 90% confidence intervals of the parameter ratios for for $AUC_{0-\infty}$, AUC_{0-t} and C_{max} as well as for differences in t_{max} are summarized in Table 4.

III. Discussion

Preventing unwanted pregnancy has been an important issue for women and their families all over the world for many hundreds of years. With the development of oral hormonal contraceptives, the so-called "Pill", in the early 1960s, women finally had access to a revolutionary method of contraception. [23,24]

Combined oral contraceptives are effective in normalizing irregular periods, reducing symptoms of premenstrual dysphoric disorder, improving acne, and allowing women to avoid having their period at inconvenient times. [25]

Combinations of drospirenone and estradiol, when compared with estradiol alone, were protective against endometrial hyperplasia. This combination was also effective in reducing menopausal symptoms, thereby elucidating improvements in health-related quality of life measures without significant adverse drug events.[26] Ethinylestradiol and drospirenone not only prevents pregnancy but also results in shorter, lighter periods, reduced cramps and a regular menstrual cycle. It also helps with some symptoms of premenstrual dysphoric disorder and helps control mild to moderate acne breakouts. [27,28]

When a new oral contraceptive formulation is developed, it is crucial to ensure optimum hormone exposure during concomitant therapy with other substances, while also guaranteeing the lowest dose to prevent pregnancy and avoid side effects. To enable testing that can deal with these concerns a highly sensitive analytical method with a low limit of quantification (LLOQ) is required to accurately measure oral contraceptives concentrations in human plasma samples.

Immunoassay methods have been the most sensitive analytical procedures available for the determination of estrogens in biological samples for many years. [29,30] These methods are sensitive, but are time consuming and prone to cross reactivity by steroids and their metabolites. Gas chromatographic coupled to mass spectrometric (GC-MS) methods typically employ some type of extraction, and one or multiple steps of derivatization.[31-34] Recently, liquid chromatography with tandem mass spectrometric (LC-MS/MS) detection has been applied for the quantitative analysis of estrogens in environmental and biological samples.[35-41] LC-MS/MS is superior to immunoassay methods or GC/MS in terms of simplicity, sensitivity, selectivity and analytical throughput.

The LC–MS/MS method described here is specific due to the inherent selectivity of tandem mass spectrometry is in accordance with both Food and Drug Administration (FDA) and the National Sanitary Surveillance Agency (ANVISA) requirements for pharmacokinetic studies. This method offers the advantage over those previously reported using LC–MS/MS [35,38,40,42,43], showing a low validated LOQ 1 pg mL^{-1} (ethinylestradiol) and LOQ 250 pg mL^{-1} (drospirenone).

The mean ratio of parameters C_{max} and AUC_{0-t} and 90% confidence intervals of correspondents were calculated to determine the bioequivalence. The point estimator and the 90% confidence intervals for the AUC_{0-t} ratio (test/reference: 92.17% [89.13% - 95.32%]) indicate high similarity of both formulations with respect to the extent of ethinylestradiol exposure. A high degree of similarity was also observed for C_{max} of ethinylestradiol, as the point estimator and the 90%

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confidence interval for the C_{max} ratio are 92.16% (88.13% - 96.38%). Regarding the AUC_{0-t} ratio of drospirenone, the point estimator is 98.24% and the 90% confidence interval 94.50% - 102.12%. Furthermore, exchangeability of both formulations is also suggested by the point estimator and 90% confidence of C_{max} of this active agent (102.80% [95.11% - 111.11%]).

The AUC_{0-t} and AUC_{0-inf} are both recognized as an uncontaminated measurement of the extent of absorption. The present study showed that 90% CI of mean AUC_{0-t} and AUC_{0-inf} (after log-transformation of individual ratios) were included into the bioequivalence range (80-125%), consequently, the two formulations of ethinylestradiol and drospirenone are equivalent for the extend of absorption.

The statistical comparison of Cmax, AUC0-t and AUC0-inf clearly indicated no significant difference in the two formulations of ethinylestradiol and drospirenone. 90% confidence intervals for the mean ratio (T/R) of Cmax, AUC0-t and AUC0-inf were entirely is in accordance with both acceptance range the Food and Drug Administration (FDA) and the National Sanitary Surveillance Agency (ANVISA). Based on the pharmacokinetic and statistical results of this study, we can conclude that ethinylestradiol and drospirenone (Test Formulation) is bioequivalent a formulation reference, and that then the test product can be considered interchangeable in medical practice.

IV. Methods

a) *Study subjects*

Thirty six healthy female volunteers were selected for the study. All volunteers were healthy as assessed by physical examination, gynecological examination, electrocardiogram (ECG), oncotic cytology (Papanicolaou) and the following laboratory tests: blood glucose, urea, creatinine, uric acid, alanine and aspartate aminotransferases (ALT and AST), gamma-gluthamil transferase (γ-GT), alkaline phosphatase, total billirubin, albumin and total protein, trygliceride, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts, red blood cell counts, platelet counts and routine urinalysis. All subjects were negative for human immunodeficiency virus, and B (except for serological scar) and C hepatitis virus.

b) *Study procedures*

All subjects gave written informed consent and the study was conducted in accordance with the revised Declaration of Helsinki, the rules of Good Clinical Practice (ICH-GCP) and the Resolutions No. 196/96 and 251/97 of National Health Council – Health Ministry, Brazil. The clinical protocol was approved by the Research Ethics Committee of University of Campinas/Unicamp (São Paulo, Brazil) and the National Sanitary Surveillance Agency (ANVISA).

The study was a single dose, two-way randomized crossover design with a 28 days washout period between the doses. During each period, the volunteers were hospitalized at 7:00 p.m. They had the usual evening meal until 9:00 p.m., and an overnight fast (minimum of 10 hours).

The subjects were randomly assigned to one of the two treatment sequences. Each treatment consisted of a single dose of two tablets, corresponding to a dose of 0.04 mg ethinylestradiol and 6 mg drospirenone. The double of the daily dose was used, since administration of only 0.02 mg ethinylestradiol and 3 mg drospirenone tends to result in plasma concentrations that are too low for a rating of ethinylestradiol 72 h and of drospirenone 144 h after drug intake.

Both treatments were administered orally. Subjects have received 200 mL of water at room temperature with each administration. All volunteers were then fasted for 4 h following drug administration; afterwards a standard lunch was consumed. Standard snack and evening meal were provided 7-8 and 10-12 h after dosing, respectively. No other food was permitted during the confinement period. Liquid consumption was allowed *ad libitum* 2 h after drug administration. However, xanthine-containing drinks including tea, coffee, and cola were avoided.

Blood samples (06 mL) were collected by indwelling catheter into EDTA containing tubes before dosing and 15, 30, 45 min and also 1, 1.25, 1.5, 1.75, 2, 2.50, 3, 4, 6, 8, 12, 16, 24, 36, 48, 72, 96, 120, 144 h post-dosing for ethinylestradiol and drospirenone. The blood samples were centrifuged at 3.000 rpm for 10 min. at 4°C and the plasma decanted and storage at –20°C until assay for their ethinylestradiol and drospirenone content. All samples from a single volunteer were analyzed on the same day in order to avoid interassay variation. Arterial pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded just before and after drug administration at each full-hour sample collection.

c) *Chemicals and reagents*

Ethinylestradiol was purchased from United States Pharmacopea (lot number QOC162, Rockville, Maryland, USA). 17α-Ethinylestradiol-d4 was obtained from CDN Isotopes (lot number H352P54, Pointe-Claire, Quebec, Canada). Drospirenone was purchased from United States Pharmacopea (lot number F0G064, Rockville, Maryland, USA). Drospirenone-d4 was obtained from SynFine Research (lot number S-1211-081A4, Richmond Hill, Ontario, Canada). Acetonitrile, methanol, chlorobutane and hexane (HPLC grade). Ultrapure water was obtained from aMilli-Q system. Blank human blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with the anticoagulant EDTA (BD Vacutainer®, BD, Franklin Lakes, NJ, USA). Blank pooled plasma was prepared and stored at −20 ◦C until needed.

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d) Analytical method

Ethinylestradiol and Its internal standard 17α-ethinylestradiol-d4 were extracted from aliquot of human plasma by liquid-liquid extraction and derivatization. 1 chlorobutane is added to the samples. The organic phase is evaporated to dryness. The buffer solution of the derivatization and derivatization reagent are added to each sample. Samples is added to the hexane and the samples are centrifuged and vortexed adequately. The organic phase is evaporated to dryness. Samples reconstituted with reconstitution solution prepared with methanol and water type Milli-Q.

Drospirenone is extracted from aliquot of human plasma by solid phase extraction and derivatization procedure. To the plasma samples is added the internal standard working solution prepared in buffer solution. The samples are loaded on the top of activated cartridges and passed through the cartridges by gravity. The compound is eluted from the cartridge using methanol and evaporated to dryness. The methanol, catalyzing solution and the derivatization solution are added to each sample. The samples are mixed adequately and incubated for the derivatization step. The samples are evaporated to dryness and reconstituted with the reconstitution solution prepared with Milli-Q type water and acetonitrile.

e) Apparatus

The ethinylestradiol samples were injected into a Zorbax SB-C18, 4.6 x 50 mm, 3.5 µm column and a Applied Biosystems Sciex API 5000 tandem mass spectrometer. The mobile A phase was methanol–water (78:22, v/v), acetic acid glacial 0.2% (v/v), and the mobile phase B was a mixture of acetonitrile 100% and acetic acid glacial 0.2% (v/v) The chromatographic condition was a gradient mode performed at 35°C and at a flow rate of 1 mL/min. for pump n°. 1 and 0.5 mL/min. for pump n°. 2. The mass spectrometer was operated with + ESI and MRM using the optimized transitions 530.3 → 171.1 for the ethinylestradiol derivate and 534.4 → 171.1 for the 17α-ethinylestradiol-d4 derivative.

The drospirenone samples were injected into a Betasil CN column and a Applied Biosystems Sciex API 5000 tandem mass spectrometer. The chromatographic separation was performed with a gradient, at room temperature and at a flow rate of 1.000 mL/min. The mobile phase A was a mixture of water–acetonitrile (65:35, v/v), formic acid 0.1% (v/v) and the mobile phase B was a mixture of acetonitrile–water (90:10, v/v), formic acid 0.1% (v/v). The mass spectrometer was operated with + ESI and MRM using the optimized transitions 500.3 → 421.2 for the drospirenone derivate and 504.3 → 425.2 for the drospirenone-d4 derivative.

f) Calibration

The calibration range of ethinylestradiol was 1.00-200.00 pg/mL. Calibration standards with 8 concentrations (1.00, 2.00, 4.00, 20.00, 40.00, 80.00, 160.00, 200.00 pg/mL) and quality control standards with 3 concentrations (3.02, 70.42, 150.90 pg/mL) were prepared in human EDTA plasma. The calibration range of drospirenone was 250.00-100000.00 pg/mL. Calibration standards with 8 concentrations (250.00, 500.00, 2500.00, 20000.00, 40000.00, 60000.00, 80000.00, 100000.00 pg/mL) and quality control standards with 3 concentrations (751.50, 30060.00, 70140.00 pg/mL) were prepared in human EDTA plasma.

g) Method validation

Quantitation was based on determination of relationship between ethinylestradiol and drospirenone peaks areas and I.S. peaks areas. Selectivity was evaluated by extracting plasma samples of plasma from different volunteers, including a lipemic and hemolysed plasma. Recoveries of ethinylestradiol and drospirenone at the three QC concentrations and I.S. were determined by comparing peak areas of spiked plasma samples with the peak area in solutions prepared with the same nominal concentration. For precision (as relative standard deviation, R.S.D.) and accuracy (as relative error,R.E.) studies, samples were prepared at three QC and were analysed in the same day (intraday precision and accuracy), and analysed in 3 consecutive days (inter-day precision and accuracy).

The calibration curves were processed and the correlation coefficient was equal to or greater than 0.9979 (ethinylestradiol) and 0.9947 (drospirenone). In ethinylestradiol the accuracy and precision of back-calculated calibration standard concentrations ranged from 89.94-99.21% and 1.35-6.81%, respectively. In drospirenone the accuracy and precision of back-calculated calibration standard concentrations ranged from 85.27-102.84% and 0.66-3.58%, respectively. In ethinylestradiol the intra-day accuracy and precision of the quality control samples ranged from 101.68-103.07% and 3.84-4.26%. In drospirenone the intra-day accuracy and precision of the quality control samples ranged from 95.55-99.12% and 2.20-3.70%. Similar accuracy and precision values were observed during the study sample analysis.

The stability of ethinylestradiol was also evaluated in plasma samples kept at -20 °C for 221 days and after being submitted to 2 freeze-thawing cycles (24 h each cycle). The stability of drospirenone was also evaluated in plasma samples kept at -20 °C for 93 days and after being submitted to 2 freeze-thawing cycles (24 h each cycle). All samples described above were compared to freshly prepared ethinylestradiol and drospirenone samples at the same concentration level. All sample analysis were carried out in a GLP-compliant manner and in accordance with the current Brazilian Regulatory Agency (ANVISA) requirements and the US Food and Drug Administration Bioanalytical method validation guidance.

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h) Pharmacokinetics and Statistical analysis

The first-order terminal elimination rate constant (Ke) was estimated by linear regression from the points describing the elimination phase on a log-linear plot, using the software SAS® Institute (Version 9.1.3). Elimination half-life ($T_{1/2}$) was derived from this rate constant ($T_{1/2}$ = ln (2)/Ke). The maximum observed plasma concentration (C_{max}) and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the ethinylestradiol ($AUC_{0\text{-}72h}$) and drospirenone ($AUC_{0\text{-}144h}$) plasma concentration versus time curves from were calculated by applying the linear trapezoidal rule. In ethinylestradiol extrapolation of these areas to infinity ($AUC_{0\text{-}inf}$) was done by adding the value C72/Ke to the calculated $AUC_{0\text{-}72h}$ (where C72=plasma concentration calculated from the log-linear regression equation obtained for the estimation of Ke 72 hours after dose). In drospirenone extrapolation of these areas to infinity ($AUC_{0\text{-}inf}$) was done by adding the value C144/Ke to the calculated $AUC_{0\text{-}144h}$ (where C144=plasma concentration calculated from the log-linear regression equation obtained for the estimation of Ke 144 hours after dose).

The bioequivalence between both formulations was assessed by calculating individual C_{max}, $AUC_{0\text{-}t}$, $AUC_{0\text{-}inf}$ and $C_{max}/AUC_{0\text{-}t}$ ratios (test/reference) together with their mean and 90% confidence intervals (CI) after log transformation of the data. The inclusion of the 90% CI for the ratio in the 80% to 125% range was analyzed by nonparametric (SAS® Institute Version 9.1.3) and parametric (ANOVA) methods.

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VI. Conflict of Interest

The authors declared no conflict of interest.

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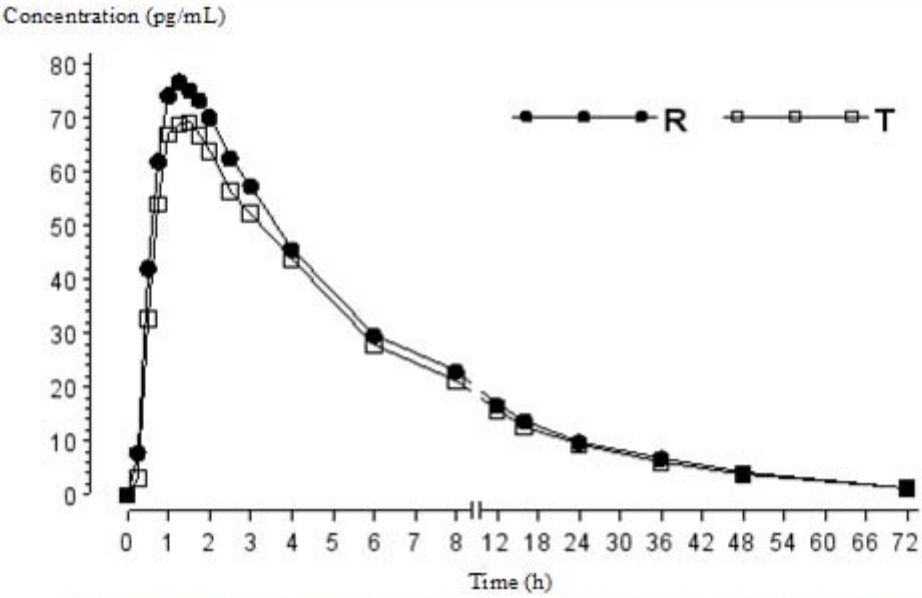

Figure 1 Mean plasma concentration-time profile of ethinylestradiol over the first 72 h after oral administration of the test and reference formulation.

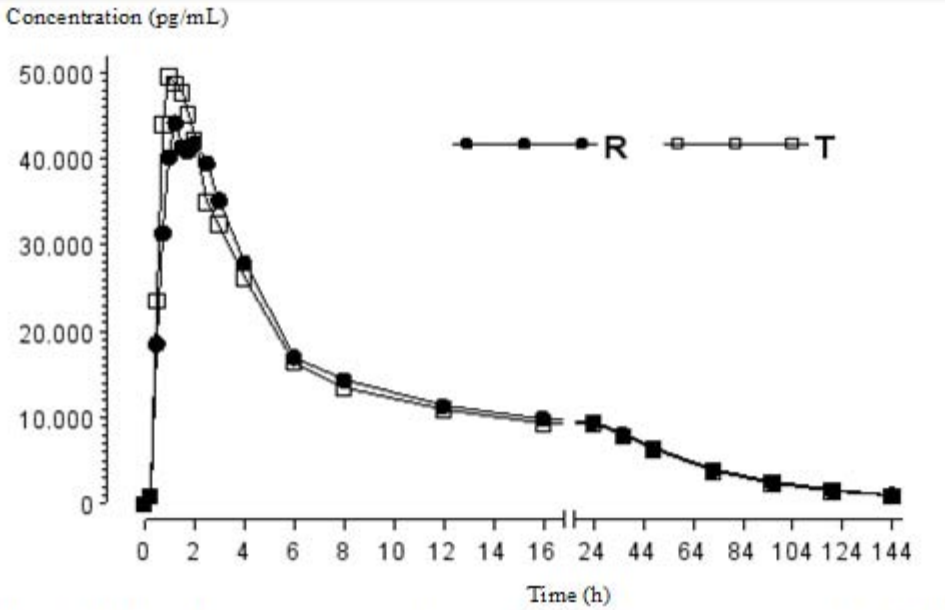

Figure 2 Mean plasma concentration-time profile of drospirenone over the first 144 h after oral administration of the test and reference formulation.

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Table 1 Summary of demographic characteristics for the safety population for study (mean ± SD)

Category	Volunteers
n	36
Age (years)	36.04 ± 6.62
Height (cm)	1.60 ± 0.06
Weight (Kg)	64.90± 8.00
BMI (Kg/m²)	25.30± 2.39

Table 2 Mean pharmacokinetic parameters of ethinylestradiol and drospirenone of test and reference formulation

	ETHINYLESTRADIOL				DROSPIRENONE			
	TEST		REFERENCE		TEST		REFERENCE	
Parameter (unit)	Mean (Median)	Standard Deviation (Amplitude)	Mean (Median)	Standard Deviation (Amplitude)	Mean (Median)	Standard Deviation (Amplitude)	Mean (Median)	Standard Deviation (Amplitude)
AUC_{0-t} (pg.h/dL)	746.06	213.77	807.17	215.27	83.5564.88	199702.16	851151.59	203231.96
$AUC_{0-\infty}$ (pg.h/dL)	794.61	226.10	854.86	221.17	88952.0.52	218454.91	906099.00	226901.38
C_{max} (pg/dL)	77.76	25.87	84.31	26.32	58431.17	10841.48	56947.08	12172.15
T_{max} (median/amp) (h)	1.25	3.25	1.25	2.50	1.00	3.25	1.25	3.50
Kel (1/h)	0.04	0.01	0.04	0.00	0.02	0.00	0.02	0.00
T½ (median/amp) (h)	17.54	12.26	16.79	7.40	34.62	31.09	35.33	37.48

Table 3 Geometric mean pharmacokinetic parameters of ethinylestradiol and drospirenone of test and reference formulation

	ETHINYLESTRADIOL		DROSPIRENONE	
	TEST	REFERENCE	TEST	REFERENCE
Parameter (unit)	Geometric Mean	Geometric Mean	Geometric Mean	Geometric Mean
AUC_{0-t} (pg.h/dL)	718.43	779.42	810015.40	824536.02
$AUC_{0-\infty}$ (pg.h/dL)	765.74	827.38	860430.42	874937.12
C_{max} (pg/dL)	74.30	80.61	57205.81	55648.61

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4 Ratios means and the 90% geometric confidence interval of test and reference formulation

	ETHINYLESTRADIOL					DROSPIRENONE				
Parameter	Ratio T/R (%)	Lower Limit (%)	Upper Limit (%)	Power (%)	Coefficient of Variation (%)	Ratio T/R (%)	Lower Limit (%)	Upper Limit (%)	Power (%)	Coefficient of Variation (%)
AUC_{0-t}	92.17	89.13	95.32	99.99	7.79	98.24	94.50	102.12	99.99	8.99
$AUC_{0-\infty}$	92.55	89.52	95.68	99.99	7.72	98.34	94.78	102.04	99.99	8.56
C_{max}	92.16	88.13	96.38	99.98	10.39	102.80	95.11	111.11	99.42	18.15
T_{max} (dif) (h)	0.25	0.00	0.50			-0.25	-0.50	0.25		

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