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A novel and sensitive method for ethinylestradiol quantification in human plasma by high-performance liquid chromatography coupled to atmospheric pressure photoionization (APPI) tandem mass spectrometry: Application to a comparative pharmacokinetics study

Ney Carter Borges[a,b,*,2], Rafael Barrientos Astigarraga[d,4], Carlos Eduardo Sverdloff[a,c,1,3], Paulo Rabelo Galvinas[d,4], Washington Moreira da Silva[d,4], Vinícius Marcondes Rezende[a,1], Ronilson Agnaldo Moreno[a,c,1,3]

[a] Synchrophar Assessoria e Desenvolvimento de Projetos Clínicos S/S Ltda, 24, Cesar Bierrenbach St., Campinas, SP 13015-025, Brazil[5]
[b] Department of Internal Medicine, Faculty of Medical Sciences, State University of Campinas, SP, Brazil
[c] Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), PB 6111, 13083-970 Campinas, SP, Brazil
[d] Magabi Pesquisas Clínicas e Farmacêuticas, 22215, Nações Unidas Av., Jurubatuba, SP 04795-100, Brazil

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ABSTRACT

In the present study, a novel, fast, sensitive and robust method to quantify ethinylestradiol in human plasma using 17α-ethinylestradiol-d4 as the internal standard (IS) is described. The analyte and the IS were extracted from acidified plasma by liquid–liquid extraction (LLE) using diethyl ether–hexane followed by online solid phase extraction (SPE) using online C18 cartridges. Extracted samples were analyzed by high-performance liquid chromatography coupled to atmospheric pressure photoionization tandem mass spectrometry (HPLC–APPI-MS/MS). Chromatography was performed isocratically on a C18, 5 μm analytical column. The method had a chromatographic run time of 2.50 min and a linear calibration curve over the range 5–500 pg ml^{-1} ($r^2 > 0.9992$). The lowest concentration quantified was 5 pg ml^{-1}, demonstrating acceptable accuracy and precision. The intra-assay precisions ranged from 2.1 to 14.6%, while inter-assay precisions ranged from 4.4 to 11.4%. The intra-assay accuracies ranged from 94.6 to 103.8%, while the inter-assay accuracies ranged from 98.9 to 101.6%. The recovery of ethinylestradiol was determined as part of the assay validation process and was 73.1 and 79.0% for the concentrations 15 and 375 pg ml^{-1}, respectively. Short-term stability showed that ethinylestradiol was stable in plasma for at least 19 h at room temperature or for at least 385 days when stored at −20 °C. In the study of bioequivalence conducted in Brazil, healthy volunteers received two ethinylestradiol 0.035 mg tablet formulations using an open, randomized, two-period crossover design with a 2-week washout interval. Since the 90% confidence interval for C_{max} and area under the curve ratios were all inside the 80–125% interval proposed by the US Food and Drug Administration, it was concluded that the two ethinylestradiol formulations are bioequivalent with respect to both the rate and the extent of absorption.

1. Introduction

17-Ethinylestradiol (EE), a synthetic estrogen developed in 1938, is an essential constituent of oral contraceptives (OC), which have been widely prescribed since the 1970s [1]. In general, ethinylestradiol is used in combination with the progestrogen 19-norethindrone (NE) or levonorgestrel (LN) to prevent pregnancy in women [2–4]. World-wide, over 60 million women currently take oral contraceptives and their safety profile is well established.

The mean bioavailability of EE is reported to be 45% [5,6]. 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 [7,8]. In addition, EE undergoes

* Corresponding author at: 24, Cesar Bierrenbach st., Campinas, SP 13015-025, Brazil. Fax: +55 19 3234 2834.
E-mail addresses: carsver@synchrophar.com, medney@synchrophar.com (N.C. Borges).
[1] Fax: +55 19 3234 2834.
[2] Tel.: +55 19 3521 8869; fax: +55 19 3521 8867.
[3] Tel.: +55 19 3251 9535; fax: +55 19 3289 2968.
[4] Tel.: +55 19 5521 0232.
[5] e-mail: synchrophar@synchrophar.com.

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sulfation and glucuronidation, resulting in the formation of EE-3-O-sulfate (EE-S) and EE-3-O-glucuronide (EE-G). EE-G and EE-S have been detected in bile, intestinal mucosa, and urine [9–11].

With the introduction of low dose contributions of OC, there has been a growing concern that their possible interaction with co-administered drugs might result in failure of contraception in women using OC. When a new OC 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) in pg ml^{-1} level for EE is required to accurately measure OC concentrations in human plasma samples.

For many years, immunoassay methods have been the most sensitive analytical procedures available for the determination of estrogens in biological samples [12,13]. These methods are sensitive, but are time consuming and prone to cross reactivity by endogenous steroids, co-administrated steroids and their metabolites. Gas chromatographic coupled to mass spectrometric (GC–MS) methods typically employ some type of extraction (liquid–liquid or solid phase), and one or multiple steps of derivatization [14–17].

The electrospray-tandem mass spectrometry (ESI-MS–MS) in negative ionization mode for ethinylestradiol has become dominant technique of their determination. Reported limits of detection (LODs) varied from 0.08 to 10 pg ml^{-1} of ethinylestradiol depending on matrix composition, method of sample preparation and model of mass spectrometer used. The method recently described by Matejicek and Kuben [18] is based on a liquid chromatographic/iontrap mass spectrometric method for the quantification of ethinylestradiol in a mixture of many others estrogenic substances in biological materials.

Recently, liquid chromatography coupled to electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) or atmospheric pressure photospray ionization (APPI)–tandem mass spectrometry has been applied for the quantitative analysis of estrogens in environmental and biological samples [15,16,18–26]. Liquid chromatography with tandem mass spectrometric detection is superior to immunoassay methods or GC/MS in terms of selectivity, sensitivity, simplicity and analytical throughput.

The APPI was introduced as an innovative ionization mode to LC–MS system in 2000 [27]. In atmospheric pressure photoionization, toluene is a dopant typically used due to its ionization potential of 8.83 eV, as well as its potential high purity grade and low toxicity [28]. It is observed in the literature data that APPI is a highly promising technique in high-throughput pharmaceutical analysis and provides superior performance in ionization of neutral compounds over electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), while providing comparable ionization for polar compounds [29].

The main objective of this study was to develop a fast, sensitive and robust method to quantify ethinylestradiol in human plasma by high-performance liquid chromatography coupled to atmospheric pressure photoionization tandem mass spectrometry (HPLC–APPI-MS/MS) using 17α-ethinylestradiol-d4 as the internal standard (IS). This method was applied to assess the bioequivalence in healthy volunteers of two low dose ethinylestradiol tablet formulations.

2. Experimental

2.1. Chemicals and reagents

Ethinylestradiol was purchased from USP (lot number QOC162, Rockville, Maryland, USA). 17α-Ethinylestradiol-d4 was obtained from Synfine (lot number A-1195-187, Richmond Hill, ON, Canada). Acetonitrile, methanol (HPLC grade) and toluene were purchased from Carlo Erba (Rodano, MI, Italy). Ethyl ether and hexane were obtained from Mallinckrodt (Phillipsburg, NJ, USA). Formic acid was purchased from J.T. Baker (London, UK). Ultrapure water was obtained from a Milli-Q system (Millipore, São Paulo, Brazil). 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 −70 °C until needed.

2.2. Calibration standards and quality control

Stock solutions of ethinylestradiol and 17α-ethinylestradiol-d4 were weighted and dissolved in pure methanol to reach a final concentration of 1.0 mg ml^{-1}. Working solutions were prepared by serial dilutions of the stock solutions in methanol–water (50:50, v/v) to obtain the final concentrations of 50, 100, 300, 600, 900, 1500, 3000 and 5000 pg ml^{-1}. Both stock and working solutions were stored at 4 °C until use. Calibration curves for ethinylestradiol were prepared by spiking blank plasma with working solutions to obtain a ten times dilution giving the final concentrations of 5, 10, 30, 60, 90, 150, 300 and 500 pg ml^{-1}. Analyses were carried out in duplicate for each concentration. Quality control samples were prepared in blank plasma at concentrations of 5, 15, 175 and 375 pg ml^{-1} (LLOQ, QCL, QCM and QCH, respectively). The spiked plasma samples (standards and quality controls) were extracted in each batch of samples.

2.3. Sample preparation

Plasma samples were thawed at room temperature. A 0.70 ml portion of human plasma sample was introduced into a glass tube followed by 0.05 ml of the IS solution (500 pg ml^{-1} of 17α-ethinylestradiol-d4 in methanol–water (50:50, v/v)) and 0.10 ml of formic acid. After vortex mixing for 10 s, 4.00 ml of ether–hexane (70:30, v/v) was added to all the tubes and extraction was effected by again vortex mixing for 40 s. Samples were centrifuged at 2000 × *g* for 2 min at 4 °C and the organic phase was transferred to another set of clean glass tubes and evaporated to dryness under N2 at 40 °C. The dry residues were dissolved in 0.50 ml of methanol–water (50:50, v/v), vortex mixed for 10 s to reconstitute the residue and transferred to 96-well plates using an automatic pipette with a disposable plastic tip. Plates were transferred to an online extraction system (Symbiosis Pharma 730, Spark Holland, Emmen, The Netherlands) and online extraction was performed as follow. First, the extraction cartridge (Hysphere C18 HD 7 μm, Spark, Holland) was activated with 1.40 ml of pure acetonitrile followed by 1.50 ml of pure methanol (at 7500 μl/min). Each cartridges was then equilibrated with 2.00 ml of methanol:water (30:70, v/v) at 7500 μl/min. The sample (0.20 ml) was injected at 2000 μl/min along with an additional 2.00 ml of methanol:water (30:70, v/v). The washing procedure was performed using 1× 1.50 ml of methanol:water (30:70, v/v) at 6000 μl/min. The sample was finally eluted with a solution of methanol:water (75:25, v/v) for 15 s.

2.4. Chromatographic conditions

After elution from the SPE cartridge, samples were injected into a Phenomenex (Torrance, CA, USA) Gemini® C18, 5 μm (4 mm × 3 mm i.d.) guard-column followed by Phenomenex Gemini® C18, 5 μm analytical column (50 mm × 4.6 mm i.d.) operating at room temperature. The mobile phase was methanol–water (75:25, v/v) at a flow rate of 0.95 ml min^{-1}. Under these conditions, typical standard retention times were 1.80 ± 0.03 min for both

ethinylestradiol and 17α-ethinylestradiol-d4, and back-pressure values of 725-1015 PSI were observed. The autosampler was maintained at 6 °C and the run time was 2.50 min.

2.5. Mass spectrometer conditions

MS detection was performed in the positive APPI mode on an Applied Biosystems Sciex API 5000 tandem mass spectrometer (Concord, Ontario, Canada) equipped with a Sciex PhotoSpray source using toluene as dopant. Interface parameters and the dopant flow rate were optimized during infusion of the ethinylestradiol and 17α-ethinylestradiol-d4 through the interface connected with the LC system and were as follows: declustering potential −80 V, heater temperature 380 °C, ion transfer voltage 800 V, curtain gas 10.0 Arbitrary Units, collision gas 10.0 Arbitrary Units and dwell time 0.2 s for each transition. Tandem mass spectrometric analysis was performed using nitrogen as the collision gas and collision energy at −44 eV. Dopant was pumped into the MS detector at a flow rate of 0.16 ml min^{-1}. Selected reaction monitoring (SRM) was used for the detection of both ethinylestradiol and 17α-ethinylestradiol-d4. The m/z 295.1 > 269.1 transition was monitored for ethinylestradiol and the m/z 299.1 > 273.0 transition for 17α-ethinylestradiol-d4 (Figs. 1 and 2). Data acquisition and analysis were performed using the software Analyst (version 1.4.2, MDS Analytical Technologies, ON, Canada).

2.6. Validation

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 [30].

2.7. Linearity

The standard calibration curves were constructed using the peak–area ratios of ethinylestradiol and IS versus ethinylestradiol nominal concentrations of the eight plasma standards (5, 10, 30, 60, 90, 150, 300 and 500 pg ml^{-1}) in duplicate. Linear least-square regression analysis, with weighting factor of $1/x$, was performed to assess the linearity, as well as to generate the standard calibration equation: $y = ax + b$, where y is the peak–area ratio, x the concentration, a the slope and b is the intercept of the regression line. In addition, a blank (non-spiked sample) and a zero plasma sample (only spiked with IS) were run to demonstrate the absence of interferences.

2.8. Ion suppression/matrix factor

A procedure to assess the effect of ion suppression on MS/MS was performed. The experimental set-up consisted of an infusion pump connected to the system by a "zero volume tee" before the spliter and the HPLC system pumping the mobile phase, which was the same as that used in the routine analysis of ethinylestradiol. The infusion pump was set to transfer a mixture of analyte and IS diluted in mobile phase to the connecting tube between the HPLC column and the mass spectrometer ion source. The concentrations of the analyte and IS mixture were selected in order to achieve at least five times the baseline. The reconstituted extract was injected into the HPLC system while the standard mixture was being infused. In this system, any ion suppression would be observed as a depression of the MS signal. The ion suppression was evaluated in normal, hyperlipemic and hemolyzed plasma samples.

Although the bioanalytical methodology herein presented uses stable isotope-labeled internal standard, we performed a matrix factor test evaluating pooled normal blank matrix sample at the low, medium and high QC level from five different sources. The samples were compared as the result of the ratio between the peak response in the presence and in the absence of the matrix ions.

2.9. Recovery

The recovery was evaluated by calculating the mean of the response of five replicates of each QCL (15 pg ml^{-1}), QCM (175 pg ml^{-1}) and QCH (375 pg ml^{-1}) concentration and dividing the extracted sample mean response by the unextracted (spiked blank plasma extract) sample mean response of the corresponding concentration. Comparison with the unextracted samples, spiked on plasma residues obtained after performing the full extraction process in blank plasma samples, was done in order to eliminate matrix effects from calculations, giving a true recovery. Since the extraction method includes an online extraction step, the unextracted samples were injected directly in the mass spectrometer, bypassing the online extraction cartridge.

2.10. Precision and accuracy

Precision and accuracy of the method were evaluated using three different batches of quality control samples at concentrations of 15, 175 and 375 pg ml^{-1} of ethinylestradiol, and the lowest limit of quantification sample, LLOQ, 5 pg ml^{-1}. Each quality control batch was evaluated in an individual analytical run. For intra-batch assay precision and accuracy, six replicates of quality control samples at the three concentration levels were assayed all at once within a day to obtain CV (%) and accuracy values. The inter-batch assay precision and accuracy were determined by analyzing mean values of quality control samples from three plasma batches, yielding the corresponding inter-batches CV (%) and accuracy values.

2.11. Sensitivity

The lower limit of quantification (LLOQ) was determined for ethinylestradiol, based on two criteria: (a) the analyte response at LLOQ had to be at least five times baseline noise and (b) the analyte response at LLOQ could be determined with sufficient precision and accuracy, i.e., precision of 20% and accuracy of 80–120%. Calculations were based on eight replicates of three blank plasma batches.

2.12. Freeze–thaw stability

Stability of ethinylestradiol was assessed in five replicates of plasma spiked with ethinylestradiol at 15 and 375 pg ml^{-1} subjected to three freeze–thaw cycles of −70 °C. In each cycle, frozen samples were allowed to thaw at controlled ambient temperature (22 °C) and were subsequently refrozen for 24 h. Aliquots of all samples were quantified at the end of the third freeze–thaw cycle. Analysis of ethinylestradiol concentrations were compared to fresh samples not subjected to the freeze–thaw cycles and expressed in percentage of degradation.

2.13. Post-processing stability.

The post-processing stability was assessed in five replicates of low and high QCs (15 and 375 pg ml^{-1}) for a 165 h period. Plasma samples spiked with QCs concentration were subjected to processing and stored after liquid–liquid extraction at room temperature prior to analyze by HPLC–MS/MS.

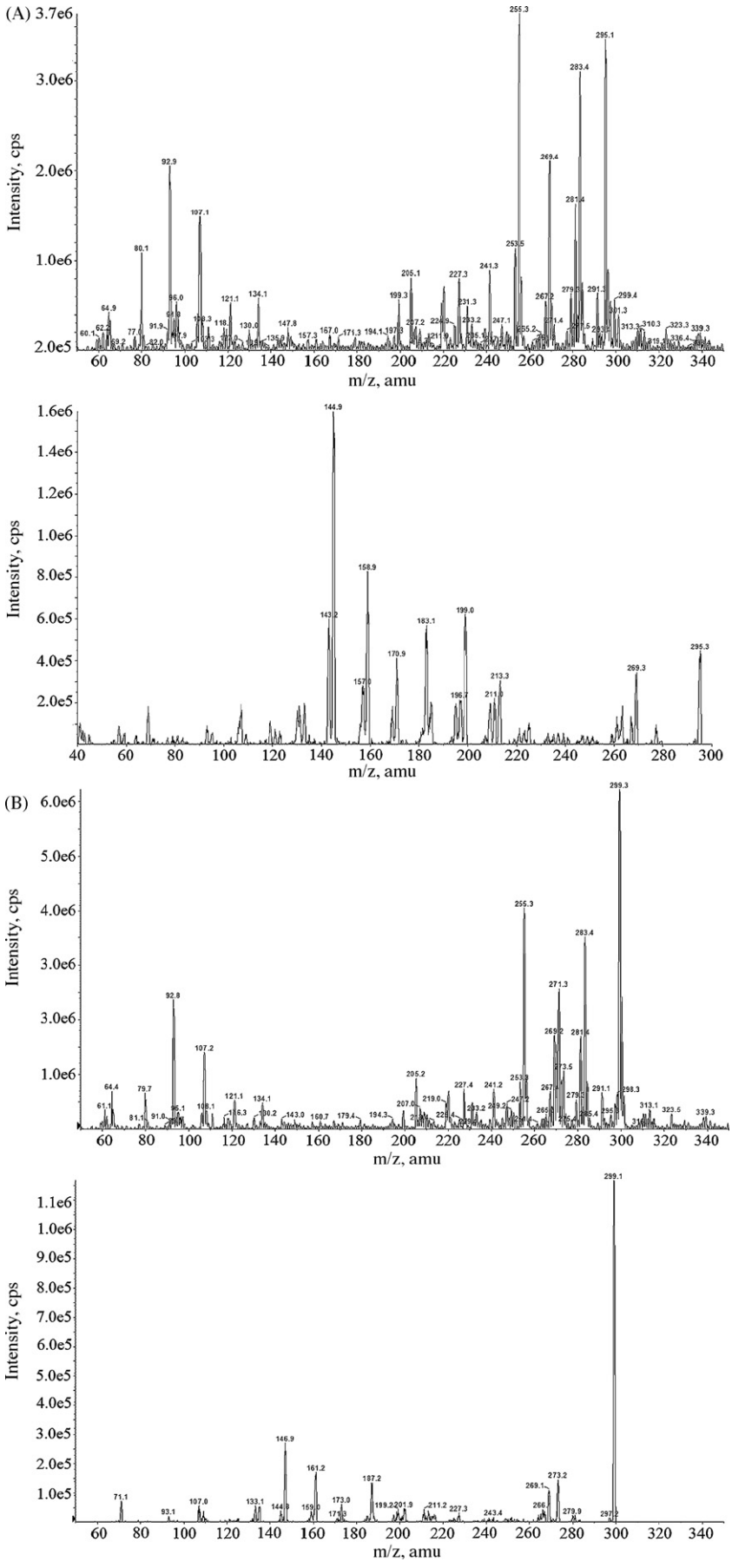

Fig. 1. Full-scan mass spectra (upper traces) and product ion spectra (lower traces) of (A) ethinylestradiol and (B) 17α-ethinylestradiol.

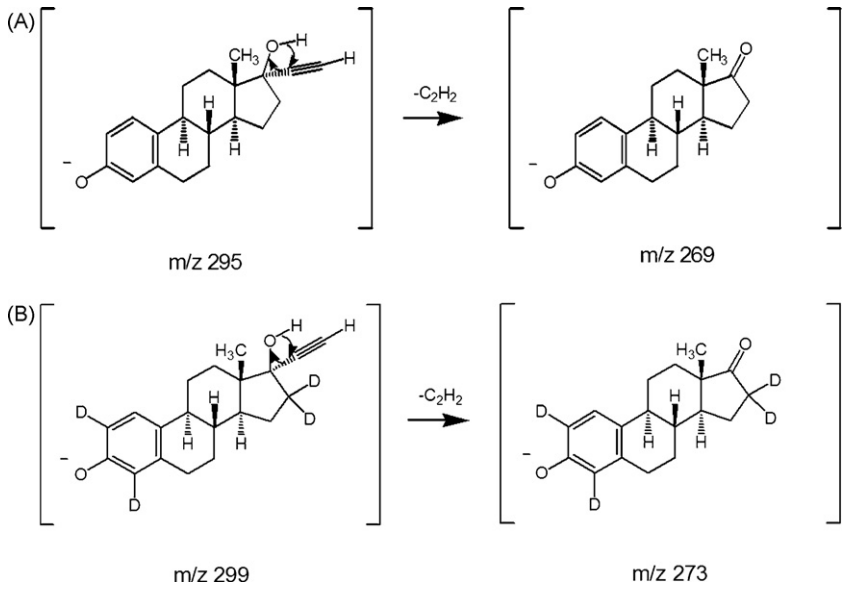

Fig. 2. Proposed fragmentation pathways for (A) ethinylestradiol and (B) 17α-ethinylestradiol-d4.

2.14. Short-term storage stability

Five replicates of low and high QCs (15 and 375 pg ml^{-1}) were thawed at room temperature (22 °C). All samples remained on the bench top for a time exceeding the maximum period of time expected for routine sample preparation (19 h). Samples were extracted and further compared to fresh prepared ones at equivalent concentration.

2.15. Long-term storage stability.

The long-term stability was assessed for five replicates of the low and high QCs (15 and 375 pg ml^{-1}) over a 385-day period. Samples were subjected to freeze storage (−70 °C) during the entire period covered by the bioequivalence study, i.e., from the first day of volunteer sample collection up to the last day of sample analysis. Storage stability was defined, comparing sample concentration to the mean values obtained during the first-day analysis.

2.16. Stock solution stability

Ethinylestradiol stock and work solutions were prepared as described and stored at 3 ± 1 °C. Sample aliquots of five replicates of low and high QCs (15 and 375 pg ml^{-1}) levels were evaluated after 20 and 42 days. Results were compared to fresh prepared solutions at corresponding concentrations.

2.17. Pharmacokinetics and statistical analysis

The analytical method developed here was applied to evaluate comparatively the ethinylestradiol plasma concentration from two tablet formulations of ethinylestradiol/cyproterone (0.035 + 2 mg) in healthy volunteers: Diane35® (lot no. 1740A, reference formulation from Shering do Brasil, São Paulo, SP, Brazil), and Selene® (lot no. 195/06, test formulation from Eurofarma Laboratorios Ltda., São Paulo, SP, Brazil).

Forty-eight healthy female volunteers aged between 21 and 45 years and index of corporal mass within 19 and 27 were selected for the study after assessment of their health status by clinical evaluation (physical examination, ECG) and the following laboratory tests: blood glucose, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, albumin and total protein, triglyceride, total cholesterol, uric acid, hemoglobin, hematocrit, total and differential white cell counts, routine urinalysis and pregnancy test βHCG. All subjects were negative for HIV, HCV and HBV. 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.

The volunteers had the following clinical characteristics expressed as mean ± SD (range): age 37.9 ± 5.6 years (21–45), height 161.0 ± 0.06 cm (148.0–177.0), body weight 60.8 ± 7.4 kg (45.1–76.0). The study was a single dose, two-way randomized crossover design with a 4 weeks washout period between the doses. The volunteers entered the Clinical Pharmacology Unit 10 h before drug administration and left the Unit 14 h after sampling. After time 0 sampling, each volunteer received a single dose of ethinylestradiol (0.035 mg of either tablet formulation) with 200 ml of water. The volunteers were then fasted for 4 h, after which period a standard lunch was served. No other food was permitted during the 'in-house' period and liquid consumption was allowed *ad libitum* after lunch (with the exception of xanthine-containing drinks, including tea, coffee, and soft drinks). The subjects were monitored throughout the study and the formulations were considered to be well tolerated. Blood samples 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, 2.33, 2.67, 3, 4, 6, 8, 12, 16, 24, 36, 48, 72, 120 h post-dosing for ethinylestradiol. The blood samples were centrifuged at 2000 × *g* for 10 min at room temperature and the plasma separated and stored in a polypropylene cryogenic screw capped tubes at −70 °C until analyzed for ethinylestradiol content.

Bioequivalence between the two formulations was assessed by calculating individual test/reference ratios for the peak of concentration (C_{max}), area under the curve (AUC) of plasma concentration until the last concentration observed (AUC_{last}) and the area under the curve between the first sample (pre-dosage) and infinite ($AUC_{0-\infty}$). C_{max} and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the ethinylestradiol plasma concentration versus time curves from 0 to the last detectable concentration (AUC_{last}) were calculated by applying the linear trapezoid rule. Statistical calculations were

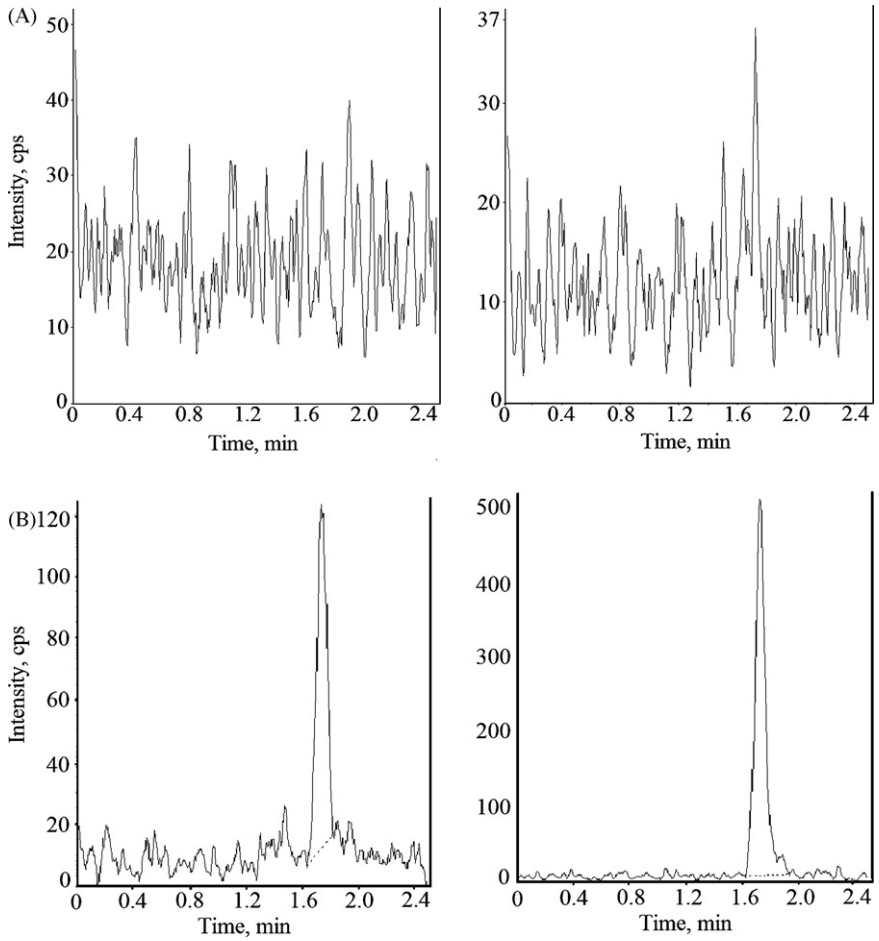

Fig. 3. MRM chromatograms of: (A) blank normal human plasma, (B) ethinylestradiol at LOQ concentration (5 pg ml^{-}1) (right panel) and internal standard 17α-ethinylestradiol-d4 (left panel) in normal plasma. The *m*/*z* 295.1 > 269.1 transition was monitored for ethinylestradiol and the *m*/*z* 299.1 > 273.0 transition for 17α-ethinylestradiol-d4.

defined at the level of $P \leq 0.10$ and bioequivalence for Diane35® and Selena® formulations was concluded if the 90.0% confidence interval for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ felt within the range of 80.0–125.0% defined by both the Food and Drug Administration (FDA) and the National Sanitary Surveillance Agency (ANVISA). The software used included Equivtest® 2.0, MS Excel® 97, Tinn-R1.1, Win-Edit® 2.0 and Scientific Work Place® 5.0.

3. Results

3.1. Linearity and specificity

The simplest regression method for the calibration curves of the ethinylestradiol was $Y = a + bx$ from 5 to 500 pg ml^{-1}. Correlation coefficient ranged from 0.9992 to 0.9998.

The chromatograms obtained from LLOQ (5 pg ml^{-1}) and extracted blank plasma are presented in Fig. 3. The ethinylestradiol and I.S. retention times were both 1.80 ± 0.03 min. The signal-to-noise ratio was higher than 7.

In the case of both ethinylestradiol and its IS, there was no significant ion suppression in the region where the analyte and internal standard are eluted. There was no suppression when the analysis was performed using blank normal plasma (Fig. 4a), and the two other batches of hemolyzed (Fig. 4b) and hyperlipemic plasma (Fig. 4c). Regarding the matrix factor, the results showed a low variability within the quality control samples analyzed. The ratios between the peak response in the presence and in the absence of the matrix ions for QCL, QCM and QCH samples were 1.10, 1.08 and 1.08, respectively.

3.2. Recovery of ethinylestradiol

Ethinylestradiol and the IS showed the recoveries (values ± CV (%), $n = 5$) for QCL, QCM and QCH as follows: 73.1 ± 20.9%, 69.1 ± 15.5% and 79.0 ± 7.3%, respectively. The recovery of the IS was 69.8 ± 18.7%.

3.3. Accuracy and precision

Intra-batch precision and accuracy of the assay was measured for ethinylestradiol at each QC level (15, 175 and 375 pg ml^{-1}) as presented in Table 1.

These results were within the acceptance criteria for precision and accuracy, i.e., deviation values were within ±15% of the nominal values, except for LLOQ, which could show a ±20% deviation.

3.4. Stock solution stability

Stability tests indicated that there was no significant degradation of the stock solution at 4 ± 2 °C. After 20 days, the variation between fresh and stored samples was 1.7 and 1.6% for low and high QC samples, respectively. In addition, the variation between fresh and stored samples after 42 days was 8.4 and −4.9% for low and high QC samples, respectively.

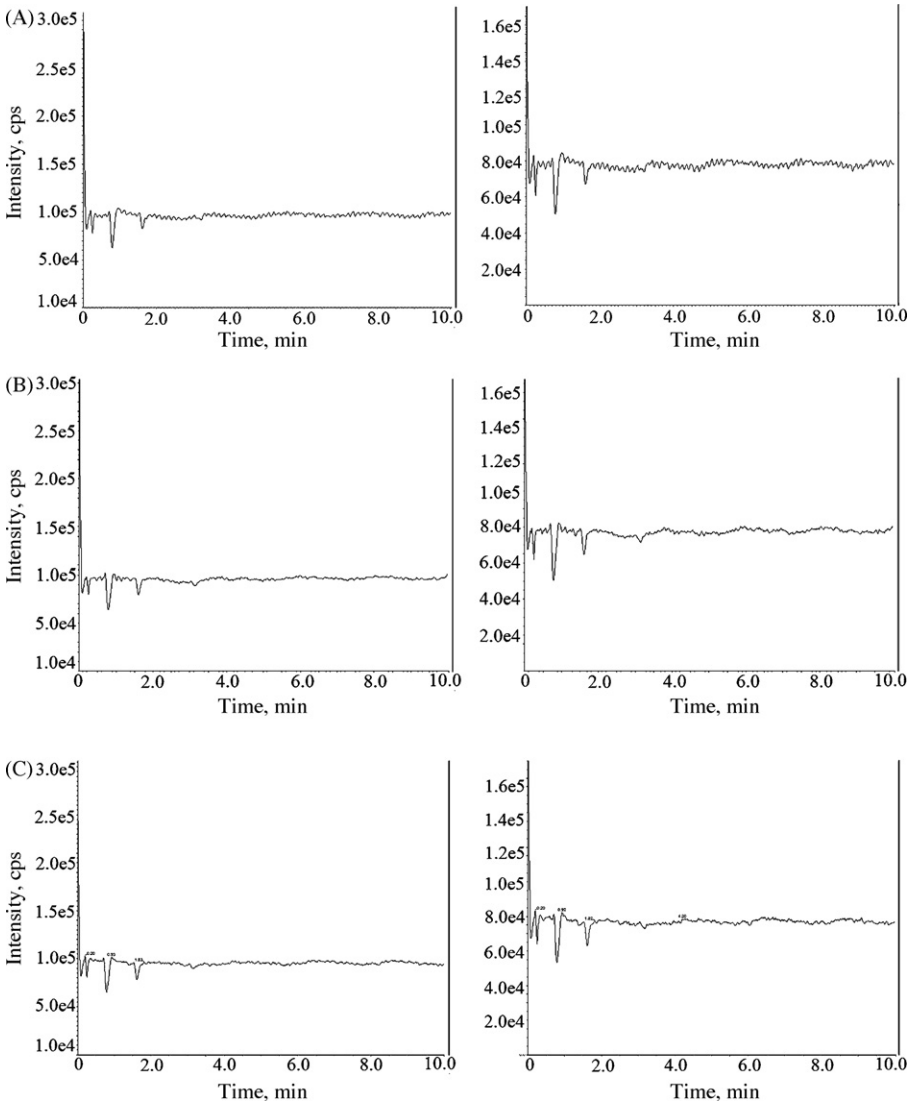

Fig. 4. Ion suppression procedure: (A) normal plasma, (B) lipemic plasma infusion and (C) hemolized plasma injection.

3.5. Stability of ethinylestradiol in human plasma

The stability of ethinylestradiol was assessed in human plasma and the steroid demonstrated no significant degradation after 19 h at room temperature, three freeze and thaw cycles, 165 h post-processing or 385 days at −70 °C (Table 2).

3.6. Comparative pharmacokinetics study

Ethinylestradiol was well tolerated at the administered doses and no significant adverse reactions were observed or reported. No clinically relevant change was observed in any measured biochemical parameter. A total of 42 volunteers finished the study. The mean ethinylestradiol plasma concentration versus time curves obtained after a single oral dose of each formulation is shown in Fig. 5. The plasma concentration of ethinylestradiol did not differ significantly after administration of both formulations (test formulation and the reference one).

Table 3 shows the values of the pharmacokinetic parameters and Table 4 summarizes the bioequivalence analysis for ethinylestradiol formulations. Briefly, the geometric mean and respective 90% CI of ethinylestradiol test/reference percent ratios were 112.20% (105.34–119.50%) for C_{max} and 92.10% (86.46–98.11%) for AUC_{0-t}.

Table 1
Accuracy and precision data for ethinylestradiol quantification in human plasma. Results were obtained during the validation of QC samples, including the LLQ in human plasma.

QC samples	Nominal concentration (pg ml^{-1})	Intra-run accuracy[a]	Inter-run accuracy[b]	Intra-run precision[c] (% CV)	Inter-run precision[b] (% CV)
QC-LLOQ	5.00	102.2	101.0	13.0	11.4
QCL	15.0	100.0	98.9	9.8	9.1
QCM	175	103.3	101.7	3.6	4.4
QCH	350	101.3	100.6	5.1	5.2

[a] ($n = 6$) expressed as (found concentration/nominal concentration) × 100.
[b] Values obtained from all three runs ($n = 18$).
[c] $n = 6$.

Table 2
Stability tests of ethinylestradiol in human plasma.

	Initial mean concentration (pg ml^{-1})	% CV	Final mean concentration (pg ml^{-1})	% CV	Variation (%)
Freeze and thaw stability test (three cycles)					
QCL	16.00	6.8	15.07	8.6	−5.8
QCH	360.39	7.7	322.23	3.5	−10.6
Short-term stability test (19 h)					
QCL	16.00	6.8	14.27	7.0	−10.8
QCH	360.39	7.7	328.41	1.9	−8.9
Post-processing stability test (165 h)					
QCL	15.06	8.8	14.12	8.4	−6.2
QCH	368.13	5.7	371.07	4.9	0.8
Long-term stability test (385 days, −70 °C)					
QCL	16.00	6.8	14.34	6.0	−10.4
QCH	360.39	7.7	349.77	5.3	−2.9

$n = 5$ for each test.
QCL = 15 pg ml^{-1}; QCH = 350 pg ml^{-1}.

Table 3
Arithmetic mean pharmacokinetic parameters obtained from 42 volunteers after administration of each 0.035 mg ethinylestradiol tablet formulation.

	Ethinylestradiol test formulation		Diane® reference formulation	
	Mean	SD	Mean	SD
C_{max} (ng ml^{-1})	84.14	26.61	74.85	23.93
T_{max} (h)	1.46	0.74	2.06	1.14
$T_{1/2}$ (h)	21.22	9.31	25.07	16.23
AUC_{last} ([ng × h] ml^{-1})	911.42	248.05	995.93	300.50
AUC_{∞} ([ng × h] ml^{-1})	1176.48	349.30	1351.72	622.33

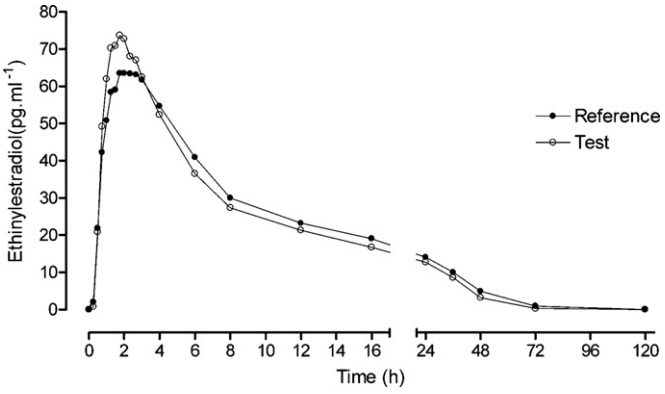

Fig. 5. Ethinylestradiol plasma mean concentration versus time profiles obtained after the single oral administration of 0.035 mg of ethinylestradiol formulations.

4. Discussion

The LC–MS/MS method described here for drug quantification is in accordance with both Food and Drug Administration (FDA) and the National Sanitary Surveillance Agency (ANVISA) requirements for pharmacokinetic studies.

The sample preparation method described in this work includes a simple liquid–liquid extraction followed by online SPE extraction. Blank plasma samples from all 25 volunteers showed a clear chromatogram in all cases. The main reason for this achievement was the improvement of clean-up obtained with the SPE procedure during the online extraction, compared to liquid–liquid extraction alone, providing a clean extracted sample and a reproducible quantification allied to the high selectivity of the MRM mode on LC–APPI-MS/MS spectrometer. This is the first method developed to assess the ethinylestradiol quantification in human plasma applied to a pharmacokinetics study using LC–MS/MS with a photoionization source. This method offers the advantage over those previously reported using LC–MS/MS [18,21,22,25], showing a low validated LOQ (5 pg ml^{-1}) associated with a faster chromatographic run time (2.5 min).

In the literature, three other methods use AAPI to quantify ethinylestradiol, but in very different matrixes. Two of them were developed to examine the hormone level in water matrixes, including the influent and effluent of wastewater treatment plants [31,32]. The third method was developed for the direct determination of ethinylestradiol in the incubation mixtures of hepatocytes to support *in vitro* hepatic clearance studies [33]. Even using the same basic APPI technique, those methods cannot be fully compared to ours based on the matrixes differences and the application of the method. The method developed by Chen et al. [31] used both the ESI and APPI ionization for the analysis of three natural estrogenic compounds (estrone, 17β-estradiol and estriol) and two synthetic estrogenic compounds (17α-ethynylestradiol and diethylstilbestrol) in the influent and effluent of wastewater treatment plants. Because of the simultaneous analysis of many compounds, the APPI method needed a very long run time of 45 min and the LOQ was only 60 pg/ml for ethinylestradiol, twelve times higher than the LOQ described in our work. The work of Lien et al. [32] is a laborious method developed for the comparison of the sensitivities and matrix effects of four ionization modes and four reversed-phase liquid chromatographic systems on analyzing seven estrogenic compounds and their derivatives of dansyl chloride or pentafluorobenzyl bromide in water matrixes. In that work, the best results were obtained with dansylated compounds using the ESI-MS/MS analysis. Indeed, these methods are not applicable for the

Table 4
Geometric mean of the individual AUC_{last}, $AUC_{0-\infty}$ and C_{max} ratios (test/reference formulation) and the respective 90% CIs.

Parameters	Parametric ($n = 42$)			
	Geometric mean (%)	90% CI	Power (%)	CV (%)
AUC_{last} (% ratio)	92.10	86.46–98.11	99	17.31
AUC_{∞} (% ratio)	89.38	81.43–98.10	98	25.75
C_{max} (% ratio)	112.20	105.34–119.50	99	17.28

pharmacokinetics study described in our work. Similarly, the method developed by Li et al. [33] for the detection of 17α-ethinylestradiol in hepatocytes could not be applied in our study system, since the LOQ was much higher and the sample preparation did not involve any extraction step. Plasma samples are much more complex than the hepatic incubation mixtures to study *in vitro* clearance. When plasma samples are extracted, the analyte need to be extracted cleanly before the chromatographic separation and APPI-MS–MS analysis. The absence of an efficient extraction method seriously compromises the sensitivity and the reproducibility of the quantification method in plasma samples.

Although it is well known that ethinylestradiol and 17α-ethinylestradiol-d4 are not stable at low pH, no perceivable degradation of either was observed under the described conditions. Therefore, we suggest that the time was insufficient for the decomposition of the analyte and IS. The method provides excellent analytical performance for ethinylestradiol extraction and proved to be appropriate for analyzing human plasma samples. The reported analytical method has been successfully applied to human pharmacokinetic investigations and bioequivalence was confirmed by the 90% confidence interval for the ratios of the C_{max} and AUC_{0-t} values being within the acceptance range of 80–125%.

5. Conclusion

This work describes a fast, sensitive and robust method to quantify ethinylestradiol in human plasma using 17α-ethinylestradiol-d4 as the internal standard. Extracted samples were analyzed by high-performance liquid chromatography coupled to Atmospheric pressure photoionization tandem mass spectrometry. This method agrees with the requirements proposed by the US Food and Drug Administration of high sensitivity, specificity and high sample throughput in comparative pharmacokinetic assays such as bioequivalence studies. The lowest concentration quantified was 5 pg ml^{-1} with suitable accuracy and precision. The intra-assay precisions ranged from 2.1 to 14.6%, while inter-assay precisions ranged from 4.4 to 11.4%. The intra-assay accuracies ranged from 94.6 to 103.8%, while the inter-assay accuracies ranged from 98.9 to 101.6%. The described method for ethinylestradiol quantification in human plasma was successfully applied in a bioequivalence study of two ethinylestradiol 0.035 mg tablet formulations using an open, randomized, two-period crossover design. Since the 90% CI for C_{max} and AUC ratios were all inside the 80–125% interval, it was concluded that the test formulation of ethinylestradiol is bioequivalent to the reference formulation with respect to both the rate and the extent of absorption.

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