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RESEARCH PAPER

Determination of Steroid Hormones in Oral Contraceptives by High-Performance Liquid Chromatography

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ABSTRACT

The aim of this research was to standardize a high-performance liquid chromatographic method for quantitative determination of steroid hormones, like ethinylestradiol (ETE), levonorgestrel (LEVO), and gestodene (GEST), in commercially available oral contraceptives (OCs). The combination ETE–LEVO was analyzed using a LiChrospher® 100 RP-8 column (5 μm, 125×4 mm) in LiChroCART®, with a mobile phase constituted of acetonitrile: water (60:40 v/v). Using the same column, ETE–GEST was analyzed with a mobile phase constituted of acetonitrile:water (50:50 v/v) at pH 7.5 adjusted with 0.02 M ammonium hydroxide. For both methods, a flow rate of 0.8 mL/min was utilized and detection was carried out at 215 nm. All analyses were performed at room temperature (24±2°C).

Calibration curves for ETE–LEVO were obtained using solutions with concentration ranges from 2.40 to 60.0 μg/mL (ETE), and from 12.0 to 300.0 μg/mL (LEVO). Calibration curves for ETE–GEST were obtained using solutions with concentration ranges from 2.40 to 60.0 μg/mL (ETE), and from 9.0 to 160.0 μg/mL (GEST). Correlation coefficients obtained were from 0.9999 to 0.9990. Coefficients of variation for samples containing ETE–LEVO were 0.47% and 0.38%, respectively. For samples with ETE–GEST they were 0.39% and 0.44%, respectively. The average recovery for samples with

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ETE–LEVO was 103.46% and 100.78%, respectively. For samples containing ETE–GEST it was 100.89% and 101.03%, respectively.

Key Words: *Steroid hormones; Oral contraceptives; High-performance liquid chromatography; Pharmaceutical preparations*

INTRODUCTION

Oral contraceptives (OCs) are pharmaceutical formulations containing steroid hormones in a relatively small amount. The prolonged use of the hormones offers long-term risks that are related to dosis and to individual susceptibility. These facts argue the need for precise methods of quantitative determination in pharmaceutical preparations. Some analytical methods for these quantitative determinations are indicated in the scientific literature. Methods like colorimetry (1–4), ultraviolet (UV) spectrophotometry (5–8), and fluorimetry (9–12) are subjectives and thus susceptible to many interferences. The study of new methods is needed because colorimetric methods, for example, are mainly used for qualitative tests and UV spectrophotometry is susceptible to many interferences like those of excipients, degradation products, and impurities. Fluorimetry does not allow simultaneous determination of hormones. When a gas chromatographic method (13,14) is used, a derivatization is always needed. Radioimmunoassay (15–18) methods do not present the selectivity needed for the quantitative determination of hormones. All methods above have being proposed, but are also vulnerable to significant error and did not allow the determination of hormones in association. High-performance liquid chromatography (HPLC) is a very efficient method that enables quantitative determination of these substances (19–26). The aim of this research was the standardization of HPLC methods for quantitative determination of associations of ethinylestradiol (ETE)–levonorgestrel (LEVO) and ETE–gestodene (GEST) in commercially available oral contraceptives (Fig. 1).

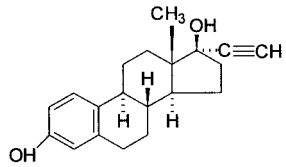
EXPERIMENTAL

Chemicals

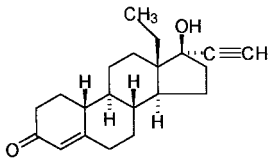
All reagents and solvents were of analytical grade and acetonitrile was HPLC grade. Water was purified by Milli-Q® Plus, Millipore System. Mobile phases were acetonitrile:water (60:40 v/v) and acetonitrile:water (50:50 v/v) at pH 7.5 adjusted with 0.02 M ammonium hydroxide solution. Solutions and mobile phases were prepared fresh each day. All solvents and solutions for HPLC analysis were filtered through a membrane filter (Millipore® Millex HV filter units, Durapore® PVDF, polyethylene, 0.45 μm pore size) and vacuum degassed before use. Steroid hormones ETE (96.9%), LEVO (98.8%), and GEST (99.7%) were donated by pharmaceutical industries and used as standards without further purification.

Instrumentation

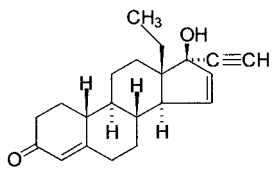
High-performance liquid chromatographic separations were made on a system consisting of a solvent delivery pump (model 480C, Instrumentos



ETE



LEVO



GEST

Figure 1. Chemical structure of ETE, LEVO, and GEST.

Científicos CG Ltd., São Paulo, Brazil) with a rotative valve injector (with a loop of 20 μL), variable UV detector (model 435, Instrumentos Científicos CG Ltd., São Paulo, Brazil). An integrator (model CG-200, Instrumentos Científicos CG Ltd., São Paulo, Brazil) was used to estimate peak areas. The column used was a Merck LiChrospher® 100 RP-8 (5 μm, 125×4 mm) in LiChroCART®.

Samples

Commercially available samples such as tablets containing 30 μg of ETE and 150 μg of LEVO/tablet (sample 1) and coated tablets containing 30 μg of ETE and 75 μg of GEST/tablet (sample 2) were used in this research.

Calibration Curves

The calibration curves were constructed by analyzing in triplicate 10 different concentrations of standard solutions containing ETE and LEVO, and ETE and GEST. Thus, stock solutions of ETE (120.0 μg/mL) and LEVO (600.0 μg/mL) were prepared separately in mobile phases. A 25.0-mL aliquot of each solution was transferred to a 50-mL volumetric flask. The resulting solution contains 60.0 μg/mL ETE and 300.0 μg/mL LEVO. Serial dilutions were made in order to obtain solutions containing ETE (2.40 to 60.0 μg/mL) and LEVO (12.0 to 300.0 μg/mL). A similar procedure was adopted for ETE–GEST. The stock solution was diluted with mobile phase to obtain solutions containing 3.20 to 64.0 μg/mL ETE and 8.0 to 160.0 μg/mL GEST.

Sample Preparation

Twenty tablets or coated tablets were weighed individually and the mean weight was determined. All the 20 units were triturated and a quantity of powder equivalent to 10 units (300.0 μg ETE and 1500.0 μg LEVO, and 300.0 μg ETE and 750.0 μg GEST) was used for sample preparation. The powder was transferred to a centrifugation tube and after the addition of 10.0 mL of acetonitrile the mixture was ultrasonicated for 20 min, followed by centrifugation at 3000 rpm for 15 min. The resulting solution was filtered through a HV Millex polyethylene filtration unit Durapore® 0.45 μm×13 mm, and 20 μL of the resulting solution injected into the chromatograph. Three injections of the mixed standard solutions and 10 of each sample solution were injected into the HPLC system in order to calculate the coefficients of variation.

Recovery Tests

Recovery tests were performed by adding a known concentration of standard solution to samples followed by analysis using the proposed methods. In brief, an amount of standard ETE equivalent to 10.0 mg and LEVO equivalent to 25.0 mg was accurately weighed and transferred to two separate 10-mL volumetric flasks. The volumes were completed with acetonitrile and the resulting solutions were ultrasonicated for 20 min. The final solutions contained 200.0 μg/mL ETE and 1000.0 μg/mL LEVO, respectively. The above solutions were mixed in equal volumes and the final solution contained 100.0 μg/mL ETE and 500.0 μg/mL LEVO. A similar procedure was followed in order to obtain a standard solution of ETE and GEST in a concentration of 100.0 μg/mL ETE and 250.0 μg/mL GEST. Appropriate amounts of these solutions were added to the sample solution according to the schematic representation in Table 1.

RESULTS AND DISCUSSION

The chromatogram representing the separation of ETE and LEVO (sample 1) can be observed in Fig. 2. Figure 3 shows the chromatogram of ETE and GEST (sample 2).

Calibration curves for ETE–LEVO were obtained by using solutions with concentration ranges from 2.40 to 60.0 μg/mL (ETE), and from 12.0 to 300.0 μg/mL (LEVO). The calibration curves showed good linearity, with a correlation coefficient of 0.9999 for ETE and 0.9996 for LEVO.

Calibration curves for ETE–GEST were obtained by using solutions with a concentration range from 2.40 to 60.0 μg/mL (ETE) and from 9.0 to 160.0 μg/mL (GEST). The correlation coefficients were 0.9992 and 0.9990, respectively.

System suitability tests are an integral part of a liquid chromatographic method. Data obtained for ETE–LEVO are presented in Table 2.

In order to standardize an efficient method for analysis of pharmaceutical formulations containing

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Table 1

Volumes of Sample and Standard Solutions Used to Perform Recovery Test

Volumetric Flask (10 mL)	Added Volume (mL)		
		Standard	
	Sample (1 and 2)	ETE (100.0 μg/mL) and LEVO (500.0 μg/mL)	ETE (100.0 μg/mL) and GEST (250.0 μg/mL)
1	1.0	—	—
2	1.0	1.0	1.0
3	1.0	2.0	2.0
4	1.0	3.0	3.0
5	—	1.0	1.0

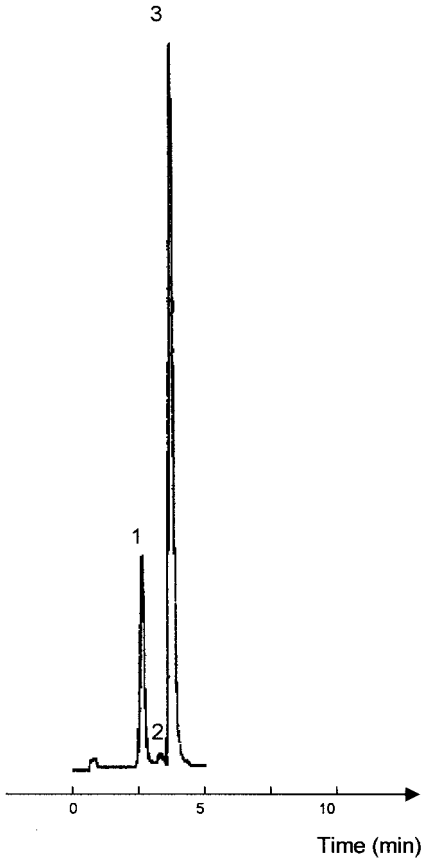

Figure 2. Chromatogram of sample 1 containing ETE (30.0 μg/mL) and LEVO (150.0 μg/mL). Conditions: LiChrospher® RP-8 column (5 μm, 125×4 mm) in LiChroCART®, mobile phase MeCN:H_2O (60:40 v/v), flow rate 0.8 mL/min, UV detection at 215 nm, and ambient temperature 24±2°C. (1) ETE (RT = 2.81 min), (2) impurity, and (3) LEVO (RT = 3.85 min).

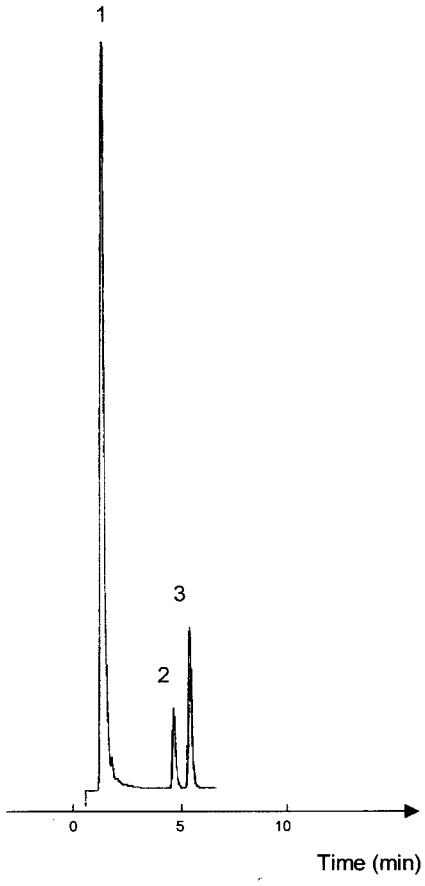

Figure 3. Chromatogram of sample 2 containing ETE (30.0 μg/mL) and GEST (75.0 μg/mL). Conditions: LiChrospher® RP-8 column (5 μm, 125×4 mm) in LiChroCART®, mobile phase MeCN:H_2O (50:50 v/v) pH 7.5, flow rate 0.8 mL/min, UV detection at 215 nm, and ambient temperature 24±2°C. (1) Excipients, (2) ETE (RT = 4.85 min), and (3) GEST (RT = 5.72 min).

OCs, preliminary tests were performed in order to choose the adequate wavelength for UV detection, the ideal proportion of solvents used as mobile phases, the proper pH, and the concentration of the standard solutions.

For the determination of the absorption spectra, standard solutions containing 10.0 μg/mL LEVO and GEST in acetonitrile were used. The UV spectra were obtained and defined by means of the obtained absorption spectra and by analyzing the three-dimensional chromatograms developed in a chromatograph with a diode array detector. Several binary or ternary eluents were tested using different proportions of many solvents such as acetonitrile, methanol, ethanol, and water. All the mobile phases were filtered in a Millipore® filtration membrane of 0.5 μm pore size and degassed before use. The flow rates tested were those compatible with the column. Different flow rates were tested in order to get better separation and peak resolution. The interval tested was from 0.5 mL/min to 1.0 mL/min. After the preliminary tests the temperature selected for all analysis was 24±2°C. The injected volume was always 20.0 μL.

Linearity was obtained through the correspondence of areas and concentration in the calibration curve for each hormone substance, in order to define the concentration interval in which the intensity of the detector response is proportional to the concentration of the analyzed substance. The correlation coefficients indicate the linearity of the method in the studied concentration ranges.

Selectivity (α) for ETE–LEVO was 1.60 and for ETE–GEST was 1.30. The confidence interval for ETE–LEVO was 92.85%±0.11% and 107.10%± 0.51%, respectively. For ETE–GEST the same interval was 100.65%±0.09% and 103.64%± 0.29%, respectively.

The precision of an analytical method can be obtained by the coefficient of variation. In order to be considered precise, the coefficient of variation of a method should be less than 2.0%. The results of statistical data obtained in the analysis of commercially available samples are shown in Table 3. The percentage of recovery results is presented in Tables 4 and 5. The recovery tests and the percentage of recovery were performed according to the recommendations of AOAC INTERNATIONAL (27). The results obtained confirmed the accuracy of the method.

Table 2

Suitability Test Parameters Determined for ETE and LEVO Using an RP-8 Column, MeCN:H_2O (60:40 v/v) as Mobile Phase, Flow Rate of 0.8 mL/min, UV Detection at 215 nm, and Room Temperature (24±2°C)

Parameter	Data
t_0 (cm)	0.20
t_A (cm)	0.70
t_B (cm)	1.00
Peak width [W_{b1} (cm)]	0.10
Peak width [W_{b2} (cm)]	0.10
Resolution (R)	3.00
Selectivity factor (α)	1.60
Theoretical plates (N_{ETE})	784
Theoretical plates (N_{LEVO})	1600
Capacity factor (K_{ETE})	2.50
Capacity factor (K_{LEVO})	4.00

Table 3

Statistical Representation of the Data Obtained in the Analysis of Commercially Available Samples Using HPLC with an RP-8 Column and MeCN:H_2O (60:40 v/v, Sample 1) and MeCN:H_2O (50:50 v/v, pH 7.5, Sample 2) as Mobile Phases

Sample		Declared Amount (μg/unit)	Found Amount[a] (μg/unit)	Standard Deviation	Coefficient of Variation (%)	Confidence Limit (%)
1	ETE	30	27.86	0.13	0.47	92.85±0.11
	LEVO	150	160.66	0.62	0.38	107.10±0.51
2	ETE	30	30.20	0.11	0.39	100.65±0.09
	GEST	75	77.74	0.34	0.44	103.64±0.29

[a]Average of five determinations.

Table 4

Recovery of ETE and LEVO Standard Solution Added to Commercially Available Sample (Sample 1, Tablet Containing 30.0 μg ETE and 150.0 μg LEVO) Using HPLC with an RP-8 Column and MeCN:H_2O (60:40 v/v) as Mobile Phase

Sample 1	Added Amount (μg)	Found Amount[a] (μg)	Recovery (%)
ETE	10.00	10.56	105.60
	20.00	20.73	103.65
	30.00	30.34	101.15
LEVO	50.00	50.22	100.45
	100.00	100.19	100.19
	150.00	152.58	101.72

[a]Average of three determinations

Table 5

Recovery of ETE and GEST Standard Solution Added to Commercially Available Sample (Sample 2, Coated Tablets Containing 30.0 μg ETE and 75.0 μg GEST) Using HPLC with an RP-8 Column and MeCN:H_2O (50:50 v/v), pH 7.5 as Mobile Phase

Sample 2	Added Amount (μg)	Found Amount[a] (μg)	Recovery (%)
ETE	20.00	20.58	102.90
	40.00	39.75	99.37
	60.00	60.24	100.41
GEST	50.00	58.20	104.39
	100.00	99.90	99.90
	150.00	149.70	99.80

[a]Average of three determinations.

CONCLUSION

The proposed HPLC methods enable the separation and simultaneous quantitative determination of ETE–LEVO and ETE–GEST in tablets and coated tablets, respectively. Ultraviolet detection at 215 nm was found to be suitable without any interference from tablets or coated tablets excipients and solvents. All the calibration curves obtained were found to be linear with values of correlation coefficients between 0.9990 and 0.9999. The coefficients of variation of ETE and LEVO (sample 1) were 0.47% and 0.38%, respectively, and for ETE and GEST (sample 2) 0.39% and 0.44%, respectively. Recovery tests confirmed the accuracy of the method. The preparation of samples is easy and efficient. There was no excipient interference in the method. The proposed HPLC methods are fast, precise, accurate, sensitive, and efficient.

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