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Original Article

High-sensitivity simultaneous liquid chromatography–tandem mass spectrometry assay of ethinyl estradiol and levonorgestrel in human plasma ☆

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ABSTRACT

A sensitive and simultaneous liquid chromatography–tandem mass spectrometry method was developed and validated for quantification of ethinyl estradiol and levonorgestrel. The analytes were extracted with methyl-tert-butyl ether: n-hexane (50:50, v/v) solvent mixture, followed by dansyl derivatization. The chromatographic separation was performed on a Kinetex C_{18} (50 mm × 4.6 mm, 2.6 μm) column with a mobile phase of 0.1% (v/v) formic acid in water and acetonitrile in gradient composition. The mass transitions were monitored in electrospray positive ionization mode. The assay exhibited a linear range of 0.100–20.0 ng/mL for levonorgestrel and 4.00–500 pg/mL for ethinyl estradiol in human plasma. A run time of 9.0 min for each sample made it possible to analyze a throughput of more than 100 samples per day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic and bioequivalence studies.

1. Introduction

Levonorgestrel/ethinyl estradiol is a progesterone and estrogen combination birth control pill. It works by preventing ovulation, thickening the mucus in the cervix and changing the lining of the uterus [1]. Combination oral contraceptives act by suppression of gonadotropins [2].

Levonorgestrel is rapidly and completely absorbed after oral administration (bioavailability about 100%). It is not subjected to first-pass metabolism or enterohepatic circulation. Therefore, it does not undergo variations in absorption after oral administration. Ethinyl estradiol is rapidly and almost completely absorbed by the gastrointestinal tract, but due to first-pass metabolism in gut mucosa and liver, the bioavailability of ethinyl estradiol is within 38%–48% [2].

The kinetics of total levonorgestrel is non-linear due to an increase in binding of levonorgestrel to sex hormone binding globulin (SHBG), which is attributed to increased SHBG level that is induced by the daily administration of ethinyl estradiol [2]. Levonorgestrel in serum is primarily bound to SHBG. Ethinyl estradiol is about 97% bound to plasma albumin. Ethinyl estradiol does not bind to SHBG, but induces SHBG synthesis.

The most important metabolic pathway of levonorgestrel occurs in the reduction of the D 4-3-oxo group and hydroxylation at positions 2a, 1b, and 16b, followed by conjugation. Most of the metabolites that circulate in the blood are sulfates of 3a, 5b-tetrahydro-levonorgestrel, while excretion occurs predominantly in the form of glucuronides. Some of the parent levonorgestrel also circulates as 17b-sulfate [2].

Cytochrome P450 enzymes (CYP3A4) in the liver are responsible for the 2-hydroxylation of ethinyl estradiol, the major oxidative reaction. The 2-hydroxy metabolite is further transformed by methylation and glucuronidation prior to urinary and fecal excretion. Levels of cytochrome P450 (CYP3A) vary widely among individuals and can explain the variation in rates of ethinyl estradiol 2-hydroxylation. Ethinyl estradiol is excreted into the urine and feces as glucuronide and sulfate conjugates, and undergoes enterohepatic circulation [2].

Evaluation of bioequivalence requires pharmacokinetic plotting of time–concentration profile to be accurate. A method for simultaneous extraction is required to extract analyte of interest selectively without co-extracting conjugated metabolites of these drugs which may be back-converted to the parent drug during the derivatization procedure.

A few assays have been reported for individual analysis of ethinyl estradiol and levonorgestrel in human plasma. However, they either lack the sensitivity required especially for ethinyl

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estradiol or do not meet the simultaneous estimation requirements of the proposed work [3–14]. Matějíček and Kubáň [5] reported a method for simultaneous analysis; however, the method was not optimized for estimation of ethinyl estradiol and levonorgestrel in human plasma samples. In another report, a semi-automated method for the simultaneous determination of oral contraceptives concentration in human plasma was reported with shorter run time [10]. However, the method lacked the required sensitivity level as the lower limit of quantification (LLOQ) for ethinyl estradiol was 10 pg/mL, which was almost 10% of the reported C_{max}.

The method presented has the highest extensive range of linearity 4.00–500 pg/mL (125 times) and 0.100–20.0 ng/mL (200 times) compared with the reported methods for ethinyl estradiol and levonorgestrel in human plasma. The plasma volume for sample preparation was 500 μL, which was considerably less than or similar to that in other reported methods [3–4]. The on-column loading of ethinyl estradiol and levonorgestrel at LLOQ was only 300 fg and 7.5 pg per sample injection volume respectively, which was significantly lower than that in all other reported procedures [3–14]. The proposed method was validated and its application to sample analysis was performed using the Watson LIMS software, which provided excellent data integrity, and they are essential requirements of current regulatory bodies. None of the methods and application were presented with the same. The results and discussion of the incurred sample reanalysis (ISR), which were obtained after implementing the proposed method, have not been discussed or presented in any of the reported methods [3–5].

2. Experimental

2.1. Chemicals and materials

Reference standards of ethinyl estradiol (Lot No.: Q0162; 100.0% purity) and levonorgestrel (Lot No.: F0H323; 99.3% purity) were obtained from USP (Rockville, USA). Ethinyl estradiol-D4 (97.1% purity) and levonorgestrel-D6 (99.9% purity) as internal standard (IS) were obtained from Clearsynth Labs Limited (Mumbai, India) and TLC Pharmachem Inc. (Concord, Canada), respectively. Dansyl chloride was obtained from Sigma Aldrich (Bengaluru, India). Sodium bicarbonate and sodium hydroxide of GR grade were procured from S.d. Fine Chem Private Limited (Mumbai, India). Formic acid and ammonia solution of GR grade were procured from Merck Private Limited (Mumbai, India). HPLC grade methyl-tert-butyl ether, acetone, acetonitrile and methanol were procured from J.T. Baker Private Limited (Mumbai, India). Water used in the entire analysis was prepared by the Milli-Q water purification system from Millipore (Bengaluru, India). Blank human plasma with sodium heparin as an anticoagulant was obtained from clinical laboratory Supratech Micropath (Ahmedabad, India). Blank plasma was stored at −20 °C until use.

2.2. Liquid chromatography and mass spectrometric conditions

A UFLC XR prominence system (Kyoto, Japan) consisting of an LC-20AD XR prominence pump, an SIL-20AC XR prominence autosampler, a CTO-20AC XR prominence column oven and a DGU-20A3 prominence degasser was used for setting the reverse-phase liquid chromatographic conditions. The separation of both analytes and respective internal standard (IS) was performed on a Phenomenex analytical column, type Kinetex C_{18} (50 mm × 4.6 mm, 2.6 μm). Column temperature was maintained at 30 °C in column oven. The mobile phase consisted of 0.1% (v/v) formic acid in water:acetonitrile with gradient elution from 60% to 90% of acetonitrile composition over a run time of 9.0 min. For gradient elution, the flow rate of the mobile phase was kept at 0.7 mL/min. The total chromatographic run time was 9.0 min. The autosampler temperature was maintained at 5 °C, and the pressure of the system was in the vicinity of 2000 psi.

Ionization and detection of analytes and IS were carried out on a triple quadrupole mass spectrometer, AB SCIEX API-5500 (Toronto, Canada), equipped with electrospray ionization (TIS interface of the API 5500) and operated in the positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor parent→product ion (m/z) transitions 313.3→245.3 for levonorgestrel (Fig. 1) and 319.3→251.3 for levonorgestrel-D6 as an IS (figure not shown). Ethinyl estradiol and ethinyl estradiol-D4 as an IS were quantitatively derivatized with dansyl chloride. The mass transitions for both compounds were 530.1→171.0 (Fig. 2) and 534.1→171.0 (figure not shown), respectively. The source dependent parameters maintained for all analytes were Gas 1 (Nebulizer gas): 40.0 psig; Gas 2 (heater gas flow): 60.0 psig; ion spray voltage (ISV): 5000.0 V, turbo heater temperature (TEM): 550.0 °C; interface heater (Ihe): ON; entrance potential (EP): 10.0 V; collisional activated dissociation (CAD): 8 psig; and curtain gas (CUR), nitrogen: 30 psig. Compound specific values of mass spectrometer parameters are listed in Table 1. Analyst software version 1.6.2 was used to control all parameters of liquid chromatography (LC) and mass spectrometry (MS). WATSON LIMS software version 7.3 was used for regression and final data processing.

2.3. Standard stock, calibration standards and quality control sample preparation

The standard stock solutions of ethinyl estradiol (0.1 mg/mL) and levonorgestrel (1 mg/mL) were prepared by dissolving requisite amount of them in methanol. Calibration standards and

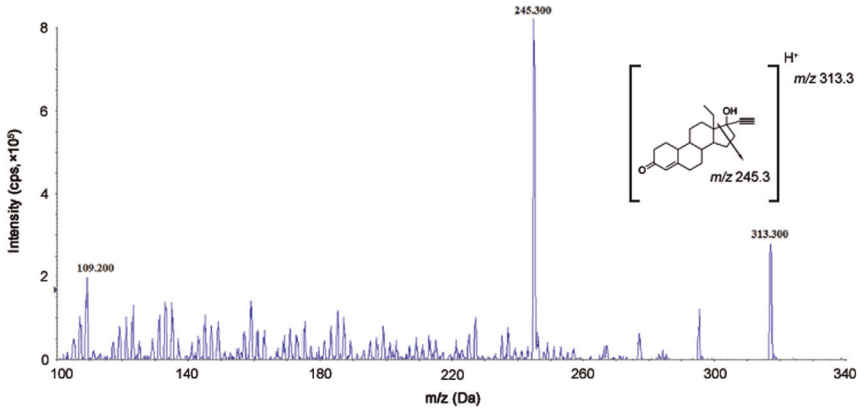

Fig. 1. Product ion mass spectra of levonorgestrel (m/z 313.3→245.3, scan range 100–350 amu) in the positive ionization mode.