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Simultaneous Determination of Etonogestrel and Ethinyl Estradiol in Human Plasma by LC-MS/MS and its Pharmacokinetic Study

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ABSTRACT:

Ethinylestradiol is an excellent drug used for the treatment of menopausal symptoms and gynecological disorders. The contraceptive vaginal ring and the etonogestrel implant are two forms of hormonal contraception that use etonogestrel. Because, it is a valuble method to develop a novel and sensitive bioanalytical approach was used for determination of pharmacokinetic studies of etonogestrel and ethinyl estradiol in rat plasma as simultaneous manner by Liquid Chromatography-Mass spectrometry. Separation was achieved on column Symmetry C_{18} column (150x4.6mm, 3.5µm) using isocratic mode with at room temperature, a mobile phase composed of FA and ACN at a ratio of 50:50 and a flow rate of 1 mL/min was made up. Ethinylestradiol was analyzed with a short retention time of 5 minutes over a good linear concentration range of 0.15ng/mL to 3ng/mL (r2 = 0.99989 ± 0.012) and etonogestrel was analyzed within 5 minutes over a linear concentration range of 1.2ng/mL to 24ng/mL (r2 =0.99955 ± 0.005).At various QC concentration levels, the extraction recoveries and matrix effects of Ethinylestradiol and etonogestrel were 98.74, 99.32, 98.12, 99.66% and 98.96, 99.18, 97.78, 99.23% for Ethinylestradiol and etonogestrel, respectively. An electrospray ionization source was used to study Ethinylestradiol and Etonogestrel at m/z 297.4032 → 60.1235, 325.4648 → 93.4218, and IS for m/z 313.4462 → 70.0059, which were ion pairs of mass analysis. This method has successfully applied to explore Ethinylestradiol (0.0003 mg/kg) with its internal standard (Levonogestrel), Etonogestrel (0.002 mg/kg) with its IS (Levonogestrel) extracted from rat plasma using LLE. The validation of the suggested technique was carried out in compliance with USFDA specifications.

Keywords: Ethinyl Estradiol, Etonogestrel, Validation, Bioanalytical Method development, LC-MS, ICH guidelines.

I. INTRODUCTION

When combined with progestins, the oestrogen drug ethinyl estradiol (EE) is a common ingredient in birth control pills [1]. In the past, EE was frequently used to treat a variety of conditions, including menopausal symptoms [2, 3], gynaecological issues, and several hormone-sensitive malignancies [4, 5]. Although it is typically taken by mouth, it can also be applied as a vaginal ring or patch [6]. Breast soreness and enlargement [7] as well as headache, fluid retention, and nausea [8] are a few of the usual adverse effects of EE. Moreover, EE can result in breast development, general feminization, Hypogonadism, and sexual dysfunction in men [9, 10]. And sexual dysfunction [11, 12], blood clots, liver damage, and uterine cancer are all rare but severe side effects. The biological target of Oestrogens like estradiol is the oestrogen receptor, and EE is an oestrogen or an agonist [14] of that receptor. It is a synthetic variant of the natural oestrogen estradiol and varies from it in a number of ways. When compared to estradiol, EE has significantly better oral bioavailability, is more resistant to metabolism, and exhibits comparatively greater effects in the liver and uterine, among other organs. In order to prevent pregnancy after sex, it is most frequently used as birth control in combination oral contraceptives (COC) [15]. In addition to being used to prevent conception, EE's birth control formulation can also be used to treat acne, menstrual symptoms, and absence of menstruation [16, 17]. Symptoms during menstruation, and acne [18, 19].

Women take a drug called etogestrel as a form of birth control. Nexplanon and Implanon are two brands that sell it as an implant that is inserted under the skin of the upper arm. NuvaRing and Circlet are two brands that sell it as a vaginal ring when combined with the oestrogen ethinylestradiol. Etonogestrel is a birth control pill having a three to four year half-life, while some research indicates a five year half-life. Fertility quickly returns after removal. Menstrual abnormalities, breast soreness, mood swings, acne, headaches, vaginitis, and other side effects are etonogestrel side effects [20]. Etonogestrel, a progestin or synthetic progestogen, is an agonist of the progesterone receptor, which is the biological target of progestogens such as progesterone. It inhibits ovulation [21], thickens the mucus around the cervix's opening, and changes the uterine lining. It has no further significant hormonal activity and has extremely mild androgenic and glucocorticoid [22] activity. Etonogestrel is a prodrug of desogestrel in the body, a closely similar and more commonly used progestin. **Figure 1** depicts the chemical compositions of etonogestrel and ethinyl estradiol.

A **B**

Fig. 1: Schematic representation of (A) Etonogestrel and (B) Ethinyl Estradiol

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

The following items were purchased from Merck India Ltd., Worli, Mumbai, India: ACN (High Performance liquid chromatography mark), Formic acid (High Performance Liquid Chromatography mark), and water (High Performance liquid chromatography mark). Ethinyl estradiol (purity 99.9%) Levonogestrel (99.9% purity) etonogestrel (purity 99.9%) APIs were purchased from Cipla Pharmaceutical Company in Mumbai.

2.2 Instrumentation and Conditions

For the bioanalytical method formulation and validation, a Waters Acquity Model LC system equipped with a QTRAP 5500 triple quadrupole mass spectrometer (SCIEX) was used. Using an isocratic model and a symmetry C18 (150x4.6 mm, 3.5m) column, chromatographic separation was performed at ambient temperature. As the mobile phase, a mixture of FA (0.1 percent) and ACN (50:50v/v) with a flow rate of 1.0mL/min was used. The injection volume was 10L, and the total run duration was 6 minutes. MS QTRAP 5500 triple quadrupole apparatus with a positive ion electrospray ionization interface was utilized for the analysis.MRM mode was used to monitor mass pair ions: m/z 297.4032 → 60.1235, m/z 325.4648 → 93.4218 for Ethinylestradiol and etonogestrel, m/z 313.4462 → 70.0059 for Levonogestrel (Internal standard of Ethinylestradiol and etonogestrel).**Table. 1** Clears details of LC-MS Instrumentation requirements.

Parameters of Liquid Chromatography		Detailed Parameters of MS	
HPLC	Waters Alliance e-2695	MS	Sciex QTRAP 5500
Isocratic mode	ACN: Formic acid 0.1% in water 50:50 v/v	Ionization source	Drying gas: N_2 gas Drying flow rate: 5 mL/min Pressure: 55 psi
	Flow level: 1 mL/min		Source temperature: 550°C
	Injection volume: 10 µL		Capillary voltage: 5500V
Waters symmetry C_{18}	150mm length	Collision cell gas	Nitrogen with high purity
	4.6 mm ID	Mode	MRM
	3.5 µm PS		
Specimens	Ethinylestradiol	Ethinyl Estradiol Multiple reaction	m/z-297.40 → m/z-60.123 CE– 15V
		monitoring transitions	
	Etonogestrel	Etonogestrel Multiple reaction monitoring transitions	m/z-325.464 → m/z-93.421 CE - 15V
IS	Levonogestrel	Levonogestrel Multiple reaction monitoring transitions	m/z-313.4462 → m/z-70.00 CE – 15V

Table.1: Optimized LC-MS Conditions

2.3 Preparation of Stock, QC and Calibration Specimens

Ethinylestradiol stock solutions at 6 ng/mL and etonogestrel stock solutions at 48 ng/mL were dissolved in FA (0.1%)-ACN (50:50, v/v), with linearity ranging from 0.15-3 ng/mL for Ethinylestradiol and 1.2-24 ng/mL for etonogestrel. The previously described working solutions were distributed and then combined with plasma to produce specimens for calibration and quality control. The concentration of eight calibration specimens were 0.15, 0.38, 0.75, 1.13, 1.50, 1.88, 2.25, 3.00 ng/mL of

Ethinylestradiol and 1.20, 3.00, 6.0, 9.0, 12, 15, 18, 24 ng/mL of Etonogestrel and In a similar manner, QC specimens were produced, with final concentrations of 0.75 ng/mL, 6 ng/mL, 1.5 ng/mL, 12ng/mL and 2.25 ng/mL, 18 ng/mL at low, medium and high. All analytes were stored at -20°C before being returned to room temperature for analysis.

2.4 Preparation of Plasma Sample solutions

For sample preparation, 200L aliquots of rat plasma specimens were contaminated with a 500L working solution of an internal standard (IS). After centrifuging the specimens for 15 minutes at 5000 rpm using centrifuging equipment, the supernatant- managed solution was split, collected, and injected into the LC system after being filtered with a 0.45µ nylon syringe filter and plasma is centrifuged at 5000 rpm for thirty minutes. Following the injection of the supernatant into the liquid chromatography column, plasma samples were maintained at a temperature ranging from 2 to 8 degrees Celsius until the finish of the analysis.

2.5 Animals Parameters

Six healthy white albino rodents weighing between 247 and 345 grammes were purchased from Biological E Limited Company Chennai, India for this study the animal ethics committee approved the protocol for the animal study. The animals were housed in controlled laboratory environments and were provided with restricted amounts of endive, carrots, and fresh maize (in limited quantities). The animals were maintained at a temperature of 21 to 24 degrees Celsius and a relative humidity of 50 to 55 percent. Before being subjected to experiments, all animals were required to abstain for one night and were given free access to water. The Pharmacokinetic evaluation of Ethinylestradiol and Etonogestrel solid dispersion tablets was performed. Ethinylestradiol and Etonogestrel were administered orally to all rodents at doses ranging from 0.12mg/kg to 0.0015mg/kg. At 0, 0.5, 1, 2, 4, 6, 8,10,12,14,16,18,24, and 30 hours, 1.5mL of blood was drawn from the rat's body in the concentration range of 12 ng/mL (Ethinylestradiol) and 1.5ng/mL (Etonogestrel), and the plasma was centrifuged at 5000 rpm for 30 minutes. The supernatant was injected into the LC column, and plasma samples were stored between 2 and 8 degrees Celsius until the analysis was complete.

2.6 Validation of developed method

2.6.1 Selectivity, Matrix effect, and Recovery

The retention times of Ethinylestradiol, etonogestrel, and IS were used to determine the selectivity of rat plasma samples from six different rodents in order to test for interference from unidentified specimens. The effects of the Matrix for Ethinylestradiol and etonogestrel were checked by looking at the percentage of peak zone in plasma samples taken from six different medication-free samples and slick recovery samples. Three times, tests were done with six different amounts of plasma at MQC levels with a level of accuracy that was allowed (15%). We found out how well Ethinylestradiol and etonogestrel were extracted by looking at each QC concentration six times. The amount of recovery was judged by comparing the highest points of different guidelines to the highest points of standards that could not be extracted.

2.6.2 Carryover and Integrity of Dilution

The purity of the dilution process was shown by injecting matrix above the upper limit of quality control with a specimen concentration and then diluting this test with blank matrix. Carryover is a term for a sample that is kept by the LC machine while another sample is injected and shows up in a series of blank or unknown analytes.

2.6.3 Precision and Accuracy

Testing was done on QC specimens (n=6) six times to get an LLOQ, LQC, MQC, and HQC level. Except for the LLOQ level, which should be less than 20%, the CV level should be less than 15%.

2.6.4 Stability

By comparing the area response of the analyte in the stability samples to the regional response of the specimen prepared from the fresh stock solution, the durability of the stock solution was determined. Experiments on plasma stability were conducted at LQC and HQC concentrations using six replicates per dose. According to USFDA regulations, the analyte was deemed stable if the variation was less than 15 percent. The stability of spiked rat plasma stored at ambient temperature for 24 hours (benchtop stability) was examined. For twenty-four hours, the stability of enriched rat plasma deposited in an

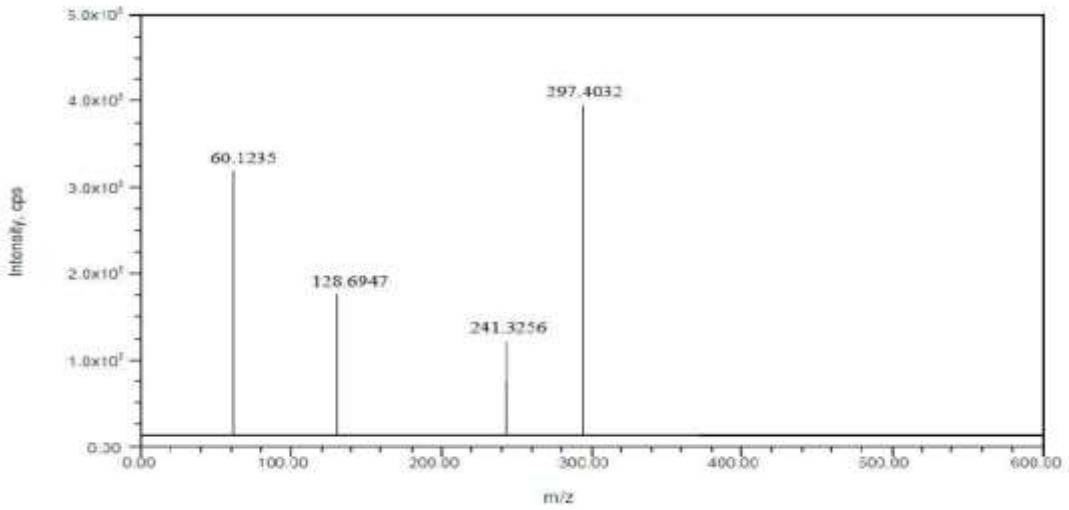
auto sampler at 2-8 °C (Autosampler stability) was evaluated. The autosampler's durability was determined by comparing extract plasma specimens that were injected promptly with samples that were stored for 24 hours at 2-8 °C in the Autosampler. The stability freeze-thaw was examined by comparing samples that were frozen at -30°C and thawed three times with freshly infused QC samples. For the evaluation of durability of freeze-thaw, six aliquots of the LQC and HQC concentration ranges were used. For evaluation of long- term durability, the concentration obtained after twenty four hours was compared with the initial concentration.

3. RESULTS AND DISCUSSION

3.1 Development of Bioanalytical Approach

In this procedure, the ESI mode with the most intense reaction over APCI mode was chosen. The Ethinylestradiol and Etonogestrel ions have been quantified using the MRM mode. Ion pair scan of Ethinylestradiol and etonogestrel m/z 297.402 and m/z 325.4648 produced [M+H] + at m/z 241.3256, m/z 128.6947, and m/z 260.0589, m/z 194.2036.Similarly, Levonogestrel internal standards scan at m/z 313.4462 and 120.5353. Ethinylestradiol and etonogestrel exhibit a favorable positive ion response mode when compared to an ion-negative mode. **Figures 2-4** illustrate the mass spectrum's specifics.

Fig.2 Mass spectrum of Ethinylestradiol



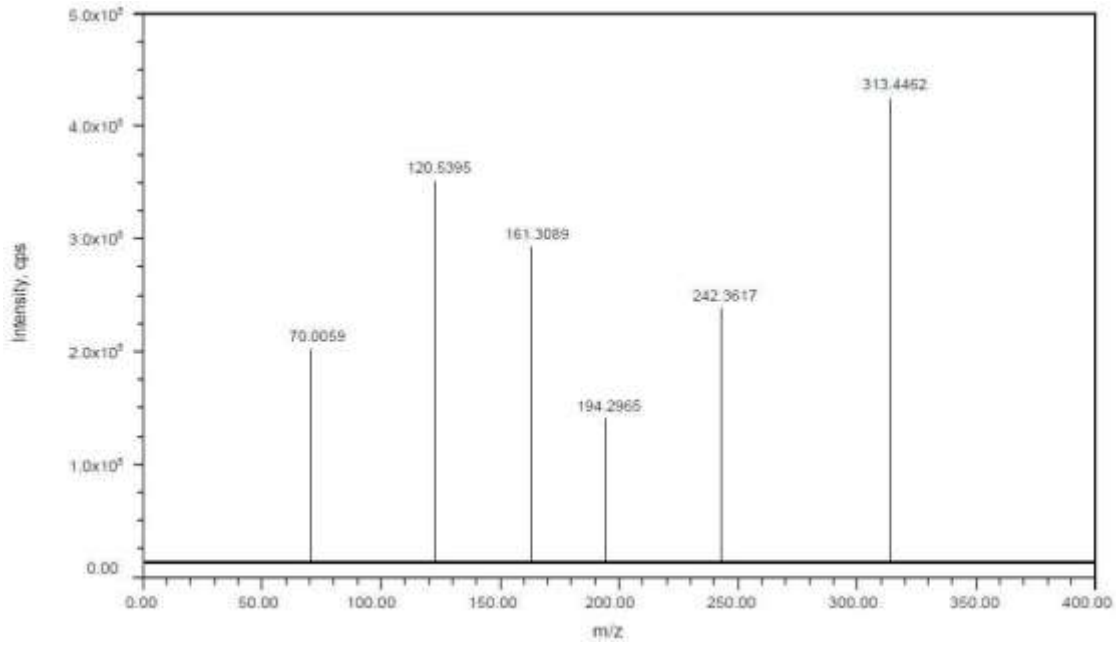


Fig. 3: Mass spectrum of Etonogestrel

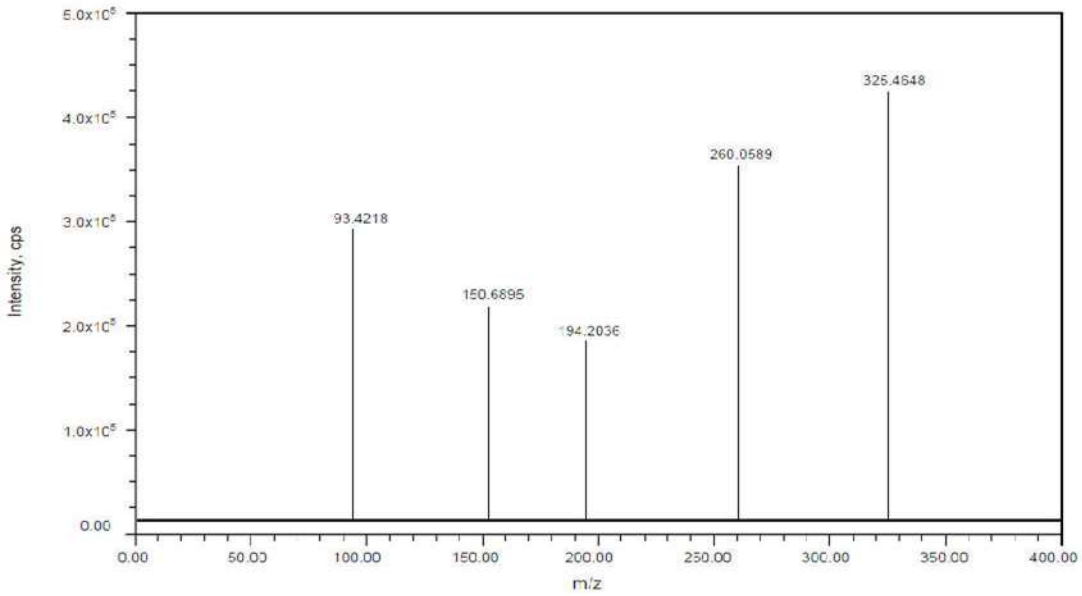


Figure.4 Mass spectrum of Levonogestrel (IS)

To get the best LC conditions, we used different buffers and different amounts of acetonitrile as the mobile phase for both isocratic and gradient modes. In each test, the mobile phase's make-up was changed to improve clarity and get good retention times. As a mobile phase, 0.1% FA and ACN in isocratic mode at 50:50 v/v ratios were chosen because they provided the greatest response from the selected medications. In the method of optimization, various stationary phases such as C18, C8, and CN-propyl were utilized. We obtained nice peak shapes for Ethinylestradiol and etonogestrel by connecting a PDA detector to a symmetry C18 column with dimensions of 150mm 4.6mm and 3.5. The discharge rates of the mobile phase were 1 mL/min. By implementing the aforementioned conditions, we determined the Ethinylestradiol and etonogestrel RTs to be 2.69 and 5.12 minutes, respectively. The percent CV of six replicate injections is 0.30. **Figures 5 and 6** depict blank and standard chromatograms, respectively.

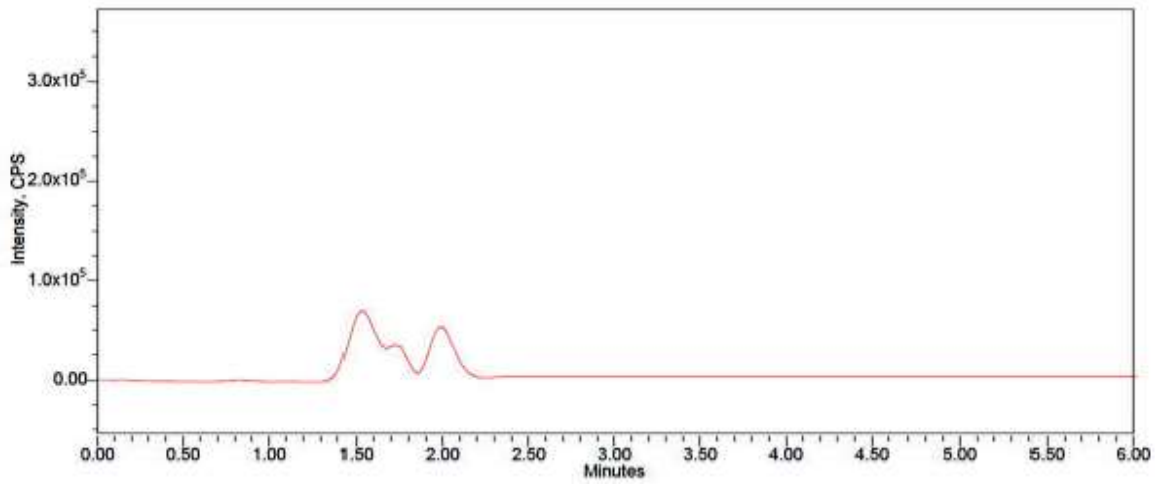
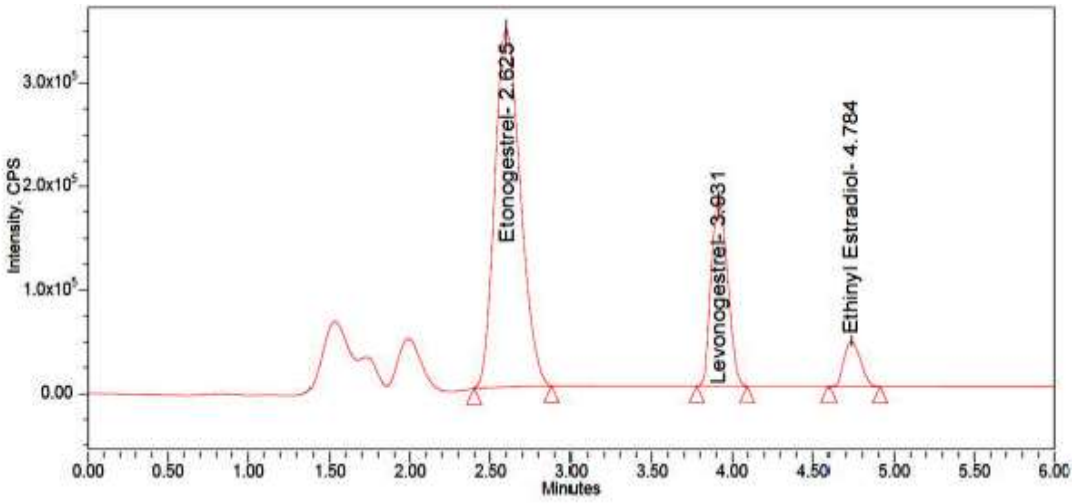


Figure.5 Chromatogram of blank plasma

Figure.6 Chromatogram of standard



3.2 Validation of Bioanalytical Process:

3.2.1 Recovery and Matrix Effect

Ethinylestradiol and etonogestrel were tested in different types of rat plasma, but they did not have a significant effect. The results that followed showed that the matrix effect on specimen ionization and internal needs were within the allowed range. Examination of recovery from Ethinylestradiol and etonogestrel at HQC, MQC, and LQC concentration focal levels in rat plasma are 0.75, 1.5, and 2.25ng/mL, and 6, 12, and 18ng/mL, which demonstrates the extraction efficiency of Ethinylestradiol and etonogestrel (**Table 2**).

Analyte	Matrix	Matrix factor bias (%)		% CV	% Recovery		% CV
		LQC	HQC		LQC	HQC	
Ethinyl Estradiol	Plasma	99.67	99.78	0.96	98.74	99.32	0.64
Etonogestrel	Plasma	98.36	99.12	1.47	98.96	99.18	0.78

Table.2: Outcomes of Matrix Variability and Recovery (%) of Ethinylestradiol and Etonogestrel in Plasma

3.2.2 Linearity, Precision, and Consistency

The region's large proportions of adjustment standards were a relative focus. The linearity range of this method for Ethinylestradiol was 0.15-3 ng/mL and 1.2-24 ng/mL for etonogestrel (Fig. **7 and 8**). The calibration curves appeared over the linear concentration range, and the CC for Ethinylestradiol and etonogestrel at various QC levels exceeded 0.9995. Ethinylestradiol and etonogestrel linearity, correlation, and precision results are shown in **tables 3, 4, 5,** and **6.**

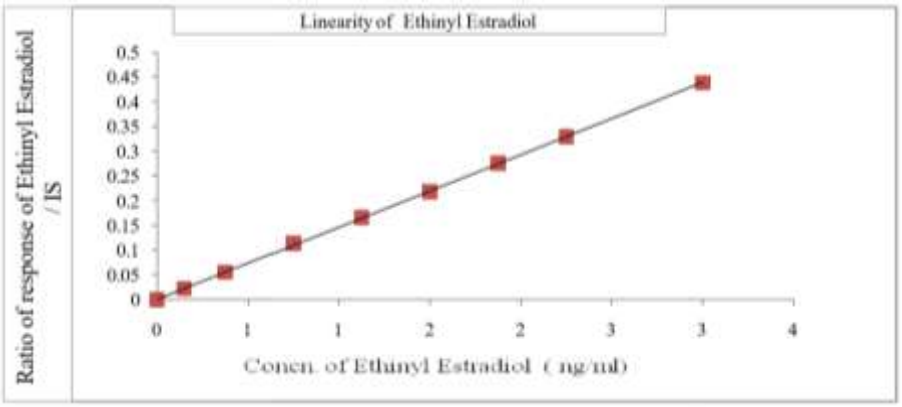


Figure .7 Calibration plot of Ethinylestradiol

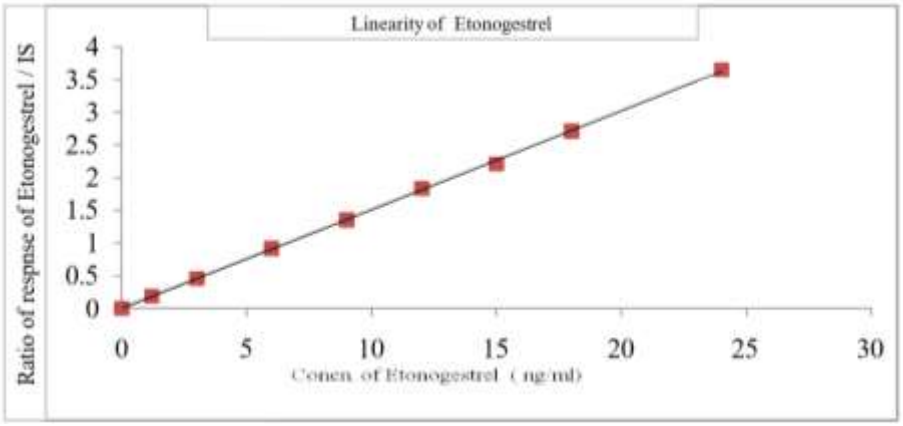


Figure.8 Calibration plot of Etonogestrel

Linearity	Ethinylestradiol conc. (ng/ml)	Ethinylestradiol area response ratio
1	0.15	0.043
2	0.38	0.107
3	0.75	0.218
4	1.13	0.323
5	1.50	0.422
6	1.88	0.531
7	2.25	0.638
8	3.00	0.854
Slope	0.1467	
Intercept	0.00038	
CC	0.99989	

Table.3: Linearity outcomes of Ethinylestradiol

Linearity	Etonogestrel conc. (ng/ml)	Etonogestrel area response ratio
1	1.20	0.356
2	3.00	0.875
3	6.00	1.754
4	9.00	2.637
5	12.00	3.554
6	15.00	4.251
7	18.00	5.253
8	24.00	7.105
Slope	0.1502	
Intercept	0.00426	
CC	0.99955	

Table.4: Linearity results of Etonogestrel

Validation parameter	Ethinyl Estradiol			Etonogestrel		
QC ranges	Low	Medium	High	Low	Medium	High
QC Conc. (ng/ml)	0.75	1.5	2.25	6	12	18
Linearity range	0.15-3 ng/mL			1.2-24 ng/mL		
Correlation (r^2)	0.99989±0.012			0.99955±0.005		

Table.5: Correlation outcomes of Ethinylestradiol and Etonogestrel

Matrix	Concentration range of specimen	Ethinyl Estradiol			Etonogestrel		
		Accuracy bias (%)	RSD of Precision (%)		Accuracy bias (%)	RSD of Precision (%)	
			Intra-day	Inter-day		Intra-day	Inter-day
Plasma	LLOQC	-0.75	1.63	1.58	-1.24	1.96	2.34
	LQC	1.02	0.79	0.63	-2.37	1.52	1.02
	MQC	0.66	0.64	0.18	-0.11	0.78	0.88
	HQC	0.37	0.52	0.76	0.26	0.65	0.57

Table.6: Precision and accuracy outcomes of Ethinylestradiol and Etonogestrel in rat plasma

3.2.3 Integrity of Dilution and Carry Over

By sprinkling the specimen matrix fixation over the Upper limit of quantification and diluting this test analyte with a blank matrix, the integrity of the dilution was demonstrated. Ethinylestradiol and etonogestrel dilution integrity was evaluated at 2ULOQC (3ng/mL to 24g/mL). Each six replicate specimens of 1:2 dilution (3.5ng/mL-12ng/mL) and 1:4 dilution (1.75ng/mL- 6ng/mL) for Ethinylestradiol and etonogestrel, and the percentage of carryover for the two components were determined to be 2.64 and 1.75, respectively, within the permissible range. The results are detailed in **table 7.**

Carryover refers to the System error that may influence the measured value of the specimen. Sample CV on a Waters Alliance- configured Liquid Chromatography-Mass system was evaluated using the following approach. Using the flow injection method, a system blank injection volume of 10L for 0.1% formic acid and acetonitrile (50:50) was conducted on a water Z-spray triple quadrupole mass detector. We can conclude that this procedure had no effect on the accuracy and precision of the proposed strategy. The results of carryover are detailed in **Table 8.**

Specimen	ULOQC conc.	Calculated conc.	%CV
Ethinyl Estradiol	3 ng/mL	2.96 ng/mL	2.64
Etonogestrel	24 ng/mL	23.88 ng/mL	1.89

Table.7: Outcomes of Dilution Integrity

Concentration	% of carryover	
	Ethinyl Estradiol	Etonogestrel
Blank	0	0
LLOQC	3.51	5.37
ULOQC	2.69	1.48

Table.8: Outcomes of carryover

3.2.4 Stability study

Ethinylestradiol and etonogestrel's stability on the benchtop was determined by preparing and storing a stock solution at room temperature for eighteen hours. In the Autosampler stability, a stock solution retained for twenty four hours at room temperature in an Autosampler demonstrates reliable stability behaviour under these conditions. To evaluate freeze-thaw stability, the stock solution was kept at (-285) °C for 24 hours. For moist extract stability, the stock solution was kept at 2-8°C for 18 hours, and for dry extract stability, the stock was kept at (-203)°C for 18 hours. The short term stability indicates

that the pharmaceuticals were stable for 7 days at (53) °C, whereas stability with long term indicates that the stock solution was stable for twenty eight days at (-203)°C and injected into the UPLC. Contrast the stability results of a freshly prepared stock solution with those of a stock solution prepared prior to 24 hours. Ethinylestradiol and etonogestrel had respective percent changes of 1.27 and 0.75, clears that solutions are stable for up to twenty four hours.

Ethinylestradiol and etonogestrel were stable in plasma under varying conditions at room temperature. At different concentration levels continued chilling and defrosting of plasma specimens spiked with Ethinylestradiol and etonogestrel were evaluated and found to have no effect on its stability. Long-term stability indicated that Ethinylestradiol and etonogestrel were stable at a capability temperature of -30°C for up to 24 hours. In **tables 9** and **10**, the overall stability results of Ethinylestradiol and etonogestrel are displayed.

Stability	**Storage condition**	**Conc. level**	**Measured conc (ng/mL) (Mean±SD, n=6)**	**% RSD**	**% Recovery**
Bench top	18 hrs at room	6	6.325±2.3	2.36	99.42
stability	temperature	12	12.142±1.8	1.47	99.26
		18	18.323±3.6	1.23	99.74
Auto sampler stability	24 hrs in auto sampler at room temperature	6	6.342±1.7	0.68	99.63
		12	12.547±2.6	0.49	99.54
		18	18.395±3.9	1.11	99.28
Long term stability	28 days at (-20±3)°C	6	6.157±2.4	0.75	99.14
		12	12.528±1.9	1.42	99.27
		18	18.452±3.2	0.34	99.36
Freeze thaw stability	24 hrs at (-28±5)°C then exposed to three freeze and thawed cycles	6	6.384±2.2	2.35	98.47
		12	12.643±4.8	2.21	99.33
		18	18.229±3.6	0.47	99.84
Wet extract stability	18 hrs at 2-8°C	6	6.347±2.1	3.31	99.17
		12	12.231±4.1	1.46	99.26
		18	18.335±2.8	0.89	99.32
Dry extract stability	18 hrs at (-20±3)°C	6	6.104±3.4	1.54	99.47
		12	12.196±2.2	1.11	99.58
		18	18.374±5.7	0.36	99.27
Short term stability	7 days at (5±3)°C	6	6.326±2.9	2.68	99.15
		12	12.374±1.7	1.74	99.64
		18	18.557±2.5	0.68	99.52

Table.9: Stability outcomes of Ethinylestradiol in plasma of rat

Stability	**Storage condition**	**Conc. level**	**Measured conc.**	**% RSD**	**% Recovery**
Bench top stability	18 hrs at room temperature	0.75	0.754±3.4	2.45	99.64
		1.5	1.569±2.5	0.79	99.35

		2.25	2.259±1.6	1.26	99.17
Auto sampler stability	24 hrs in auto sampler at room temperature	0.75	0.776±2.7	1.04	99.55
		1.5	1.526±3.2	2.58	99.42
		2.25	2.239±1.5	1.65	99.67
Long term stability	28 days at (-20±3)°C	0.75	0.773±0.7	3.64	99.28
		1.5	1.542±0.5	0.4	99.61
		2.25	2.252±1.3	1.6	99.54
Freeze thaw stability	24 hrs at (-28±5)°C then exposed to three freeze and thawed cycles	0.75	0.745±0.4	2.85	99.23
		1.5	1.525±0.9	1.64	99.78
		2.25	2.269±1.1	1.12	99.47
Wet extract stability	18 hrs at 2-8°C	0.75	0.713±2.5	2.44	99.26
		1.5	1.574±0.9	0.68	99.55
		2.25	2.216±1.7	0.12	99.64
Dry extract stability	18 hrs at (-20±3)°C	0.75	0.774±2.4	1.74	99.28
		1.5	1.569±1.8	0.44	99.45
		2.25	2.233±0.3	0.93	99.72
Short term stability	7 days at (5±3)°C	0.75	0.766±0.8	2.51	99.61
		1.5	1.497±1.7	2.01	99.84
		2.25	2.213±3.9	1.63	99.73

Table.10: Stability outcomes of Etonogestrel in rat plasma

3.3 Discussion

Due to the volatility requirement in mass spectrometry analysis, the optimized procedure exhibited excellent LC separation and mass transitions with a (50:50 v/v) mixture of acetonitrile and 0.1% formic acid. For column efficiency a Symmetry C_{18} (150mmx4.6mm, 3.5 µ) column at 30°C was used .concentration due to the requirement of its volatility in mass spectrometry analysis. For column efficiency, a Symmetry C18 (150mmx4.6mm, 3.5) column at 30°C was used. The linear regression model determines the optimal calibration curve fit for the chromatographic response versus concentration relationship. The precision and accuracy of intra- and inter-day data for Ethinylestradiol and etonogestrel values are within the acceptable range. With the optimized extraction procedure, the extraction yield was satisfactory, consistent, precise, and repeatable. The injector carry-over test revealed 0% carry-over for blank samples extracted, followed by Upper limit of quality control and Lower limit of quality control samples extracted. Studies on the freeze-thaw stability of substances frozen at -30°C and thawed three times revealed permissible limits of 85 to 115%. The sample stability of the Autosampler at 20°C for seventy hours at each of the Low quality control and High quality control concentration levels exhibited a mean percent accuracy between 85 and 115%. The percentage of accuracy was determined to be between 85 and 115%. Long-term stability was evaluated over the course of twenty eight days and compared to initial concentrations of Lower Quality Control and High Quality Control. The accuracy was determined to be between 85 and 115%. We have developed Liquid chromatography-Mass spectrometry method for the simultaneous determination of Ethinylestradiol and etonogestrel in rat plasma and its application to pharmacokinetic study under ICH guidelines [23, 24]. Few of articles were reported in the last few years for analyzing the Ethinylestradiol and etonogestrel [25-31]. In the present examination we intended to explore a specific, selective and new LC-MS method towards the analysis of Ethinylestradiol and etonogestrel.

3.4 Pharmacokinetic Study

For the purpose of determining pharmacokinetic parameters of Ethinylestradiol and etonogestrel, oral administration of 0.003 milligrams/ kg Ethinylestradiol and 0.002 milligrams/ kg etonogestrel were administered to rats in order to obtain mean plasma concentration-time profiles (**Fig.9, 10**).In pharmacokinetic investigations Ethinylestradiol and etonogestrel exhibit notable differences from one another. We collected samples from the rat's body at various times, including 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24 and 30 hrs after the medications were administered . After preparing the test sample, it was injected into the chromatographic system and the values were recorded. The C_{max} after oral administration of Ethinylestradiol and etonogestrel

(11.006 and 1.262), T_{max} (12 and 1.5 hrs), K_{el}(obvious first request terminal rate constant were calculated from semi- log plot of a plasma concentration vs time bend, using the least square relapse technique and $t_{1/2}$ (terminal half-life as governed by $0.693/K_{el}$quotient). Test/reference ratios for $C_{max,}$ AUC_{0-24}, and $AUC_{0-\infty}$ were 3964, 487.6 and 3966, 488.7 ng-hr/mL respectively, and found to be within the permissible range. The pharmacokinetic parameters of Ethinylestradiol and etonogestrel are shown in **table 11**.

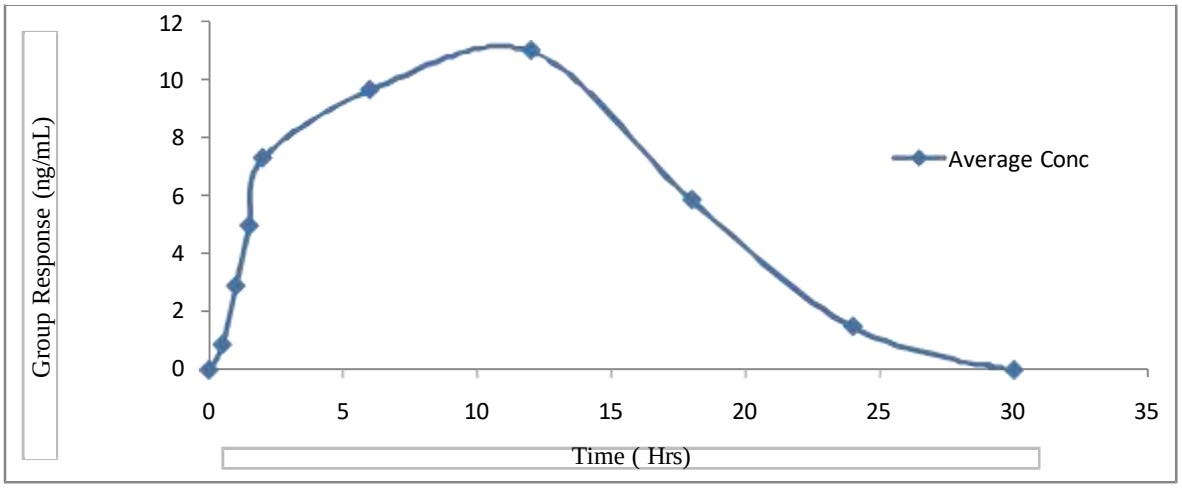


Figure9. Recovery plot of Ethinylestradiol

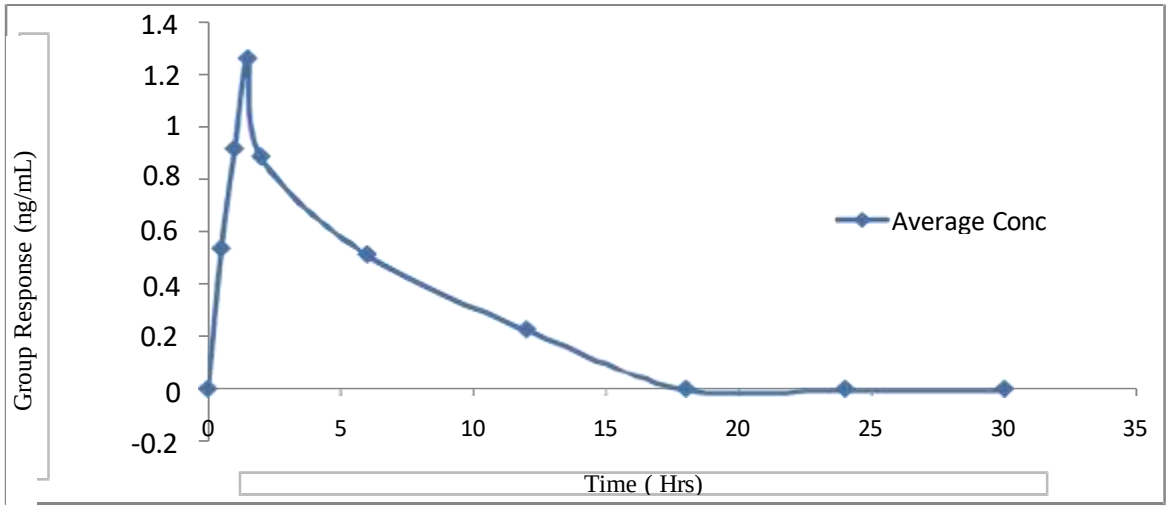


Figure.1u ĸecovery plot of Etonogestrel

Pharmacokinetic parameters	**Etonogestrel**	**Ethinyl Estradiol**
AUC_{0-t}	3964 ng-hr/mL	487.6 ng-hr/mL
Cmax	11.006 ng/mL	1.262 ng/mL
$AUC_{0-\infty}$	3966 ng-hr/mL	488.7 ng-hr/mL
tmax	12 hr	1.5 hr
T1/2	24 hr	12 hr

Table.11: Pharmacokinetic studies of Ethinylestradiol and Etonogestrel

4. CONCLUSION

It is the first time that a novel MS/LC method has been successfully devised and validated for the evaluation of Ethinylestradiol and etonogestrel in rat plasma for 5 minutes. Ethinylestradiol and etonogestrel were promptly absorbed by the rat after oral administration and exhibited pharmacokinetic

behavior; here the described method is rapid, robust, and reproducible. And it can be used successfully for PK studies and to check permissible specimen concentrations in bodily fluids accuracy and a decent linear concentration range. These studies are required to validate our findings and serve as a reference in the near future.

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