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Quantification of Ethinyl Estradiol, Levonorgestrel and Ferrous Fumarate from Bulk Drugs and Tablet Formulation using a Validated HPLC Method

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Abstract

Objective: This research article introduces new, rapid, accurate and precise HPLC method for estimating Ethinyl estradiol, Levonorgestrel and Ferrous fumarate from the bulk drug and tablets.
Methods: HPLC analysis of all the three drugs was performed on the Sun Q sil C18, (250 mm x 4.6 mm ID, 5 µm) under isocratic conditions employing mobile phase as acetonitrile: water in ratio of 70: 30 at flow rate of 0.7 mL/min with UV wavelength of 265 nm.
Results: Ferrous fumarate, Ethinyl estradiol, and Levonorgestrel had retention times of 2.86 min, 6.66 min and 9.87 min, respectively. The data from linear regression analysis studies was plotted and calibration plots demonstrated satisfactory linear connection. For Ethinyl estradiol, Levonorgestrel, and Ferrous fumarate, the mean correlation coefficient values were found to be 0.9997, 0.9992 and 0.9995. % RSD for intra-day precision and inter-day precision was found to be less than 2. Accuracy was found to be 99.58 % for Ethinyl estradiol, 99.85 % for Levonorgestrel and 100.06 % for Ferrous fumarate and the method was found to be specific.

Keywords: Ethinyl estradiol; Levonorgestrel; Ferrous fumarate; HPLC; Method development; Validation.

Abbreviations:
HPLC: High Performance Liquid Chromatography
HPTLC: High Performance Thin Layer Chromatography
RSD: Relative Standard Deviation
SD: Standard Deviation
APPI: Atmospheric Pressure Photo Ionization
MS: Mass Spectroscopy
ICH: International Council for Harmonization
API: Active Pharmaceutical Ingredient
DMSO: dimethyl sulfoxide

Introduction

Ethinyl estradiol is chemically (8R,9S,13S,14S,17R)-17-ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-diol (Figure 1) is a bio-active oral oestrogen. Ethinyl estradiol is used in practically all modern oral contraceptive pill combinations. It is one of the drugs that is taken most frequently[1]. Levonorgestrel is chemically (8R,9S,10R,13S,14S,17R)-13-ethyl-17-ethynyl-17-hydroxy-1,2,6,7,8,9,10,11,12,14,15,16-dodecahydrocyclopenta[a]phenanthren-3-one (Figure 2) is a synthetic progestogen which binds to the progesterone receptor, stimulates hormone-receptor complex, and increases protein synthesis, suppressing LH activity, ovulation inhibition, and cervical mucus and endometrium changes[2].Ferrous fumarate is chemically (E)-but-2-enedioate; iron (2+) (Figure 3), recognized as the iron (II) salt derived from fumaric acid, presents itself as a distinctive reddish-orange powder. This compound serves a dual purpose: firstly, as a means to augment iron consumption, and secondly, as a treatment for cases of iron deficiency anemia[3].

It elevates serum iron concentration, which is then assimilated into hemoglobin, required for the transport of oxygen, or trapped in the reticulo-endothelial cells for storage.The combination of these medications is used to prevent pregnancy and anemia by changing the lining of the womb and affecting sperm movement.From the literature review it was concluded that following studies have been performed; simultaneous estimation of drugs by HPTLC[4]; stability-indicating HPTLC determination of a combination[5], estimation by RP-HPLC[6], simultaneous estimation of combination by HPLC[7], estimating drugs from transdermal patches using HPLC[8], quantification of drug in plasma using HPLC together with APPI tandem MS[9].To date, no reports have been published on the concurrent estimation of Ethinyl estradiol, Levonorgestrel and Ferrous fumarate by HPLC. The current work simultaneous estimates these three drugs by HPLC for the first time. ICH guidelines were referred to perform validation[10, 11].

Materials and Methods

Materials

Ethinyl estradiol, Levonorgestrel, and Ferrous fumarate working standards were willingly provided from Hetero Healthcare Ltd., Hyderabad (Telangana, India). These medications were found to contain, on a dry weight basis, 99.89 % Ethinyl estradiol, 99.92 % Levonorgestrel, and 99.91% Ferrous fumarate. Fixed dose combination tablets Brand Name: CHOICE with Ethinyl estradiol, Levonorgestrel, and Ferrous fumarate were bought from market. All the reagents and chemicals were procured from Thomus Bakers and Loba Chemicals.

Instrumentation

The HPLC system included a Jasco PU 2080 LC pump having a 20 µL injection capacity. The PDA detector was used and the system was operated at 265 nm wavelength. The data was integrated using the LC-Net II/ADC system, Jasco Borwin version. A Sun Q sil C_{18}, (250 mm x 4.6 mm ID, 5 µm) column from Tokyo, Japan was utilized.

Preparation of Standard Stock Solutions

Std. 1

Standard stock solution of concentration each 1000 µg/mL of Ethinyl estradiol and Levonorgestrel were prepared using methanol.

↓

From these solutions a mixed standard stock solution with 5 µg/mL of Ethinyl estradiol and 25 µg/mL of Levonorgestrel was made using methanol.

Std. 2

Standard stock solution for Ferrous fumarate of concentration 1000 µg/mL was prepared using 1% nitric acid solution

↓

From this solution, 25 µg/mL of Ferrous fumarate was prepared using water. Stock solution was kept away from light at 2-8 °C.

Method Optimization
Strength of Ethinyl estradiol and Levonorgestrel is very less as compared to Ferrous fumarate in the tablet formulation. The label claim of the tablet for all three drugs is drastically different. Ferrous fumarate (60 mg) is 2000 times greater in strength than Ethinyl estradiol (0.03 mg) and 400 times greater in strength than Levonorgestrel (0.15 mg). So, it was difficult to quantitate all three drugs in one chromatogram (as per the label claim). Therefore, quantification of Ethinyl estradiol and Levonorgestrel was done together, while that for Ferrous fumarate was done separately but in the same mobile phase.For method optimization, several methanol, acetonitrile and water ratios were tried. But, acetonitrile: water (70: 30) with flow rate of 0.7 mL/min provided good peak shape with appropriate retention times, number of theoretical plates and good resolution for all three drugs. Ferrous fumarate, Ethinyl estradiol, and Levonorgestrel had retention times (Rt) of 2.86 min, 6.66 min and 9.87 min (Figure 4).

The HPLC method was optimized for developing a concurrent assay method for Ethinyl estradiol, Levonorgestrel and Ferrous fumarate. Std. 1 was injected in HPLC to analyses Ethinyl estradiol and Levonorgestrel (Figure 5). Whereas, Std. 2 was injected to analyses Ferrous fumarate (Figure 6).

Validation of the method
The parameters used to validate the optimized HPLC technique are given below.

System suitability
The crucial part of an analytical method is system suitability which ensures that the equipment, analytical operation, electronics, and samples that play an important part in the analysis are working appropriately. All the parameters pertaining to system suitability were evaluated.

Linearity
Further dilutions of Ethinyl estradiol and Levonorgestrel (1000 µg/mL each) were prepared from the standard stock solutions, yielding Ethinyl estradiol concentrations in the 5-50 µg/mL range and Levonorgestrel concentrations in the 25-250 µg/mL range.Further dilutions of Ferrous fumarate (1000 µg/mL) stock solution were prepared in concentration range between 25-250 µg/mL. Examination of linearity of method was performed by injecting 6 solutions with different drug concentration in the HPLC system in triplicate while maintaining the injection volume constant. Calibration graphs were obtained as the peak areas verses corresponding concentrations were plotted against each other.

Precision
Intraday and intermediate precision experiments were used to validate the method's precision. On the same day three different quantities of Ethinyl estradiol (5, 20, 40 µg/mL), Levonorgestrel (25, 100, 200 µg/mL), and Ferrous fumarate (25, 100, 200 µg/mL) were analysed six times each. The method's precision was tested by reperforming the analysis on three consecutive days.

Limit of detection and limit of quantitation
LOD and LOQ were determined from linearity studies. Linearity curves were generated and calibration curves were calculated for concentrations mentioned above. The regression equation was derived and the following formulas were used to calculate LOD and LOQ.

$$LOD = \frac{3.3 \times \text{Standard Deviation}}{\text{Slope}} \qquad LOQ = \frac{10 \times \text{Standard Deviation}}{\text{Slope}}$$

Robustness of the method
A few variables were intentionally altered to assess the robustness of an HPLC process. Variations in flow rate, composition of acetonitrile in the mobile phase and wavelength were amongst the parameters.

Specificity
The method's specificity was determined by analyzing the spectrum of all three drugs. The resolution factor and number of theoretical plates were examined.

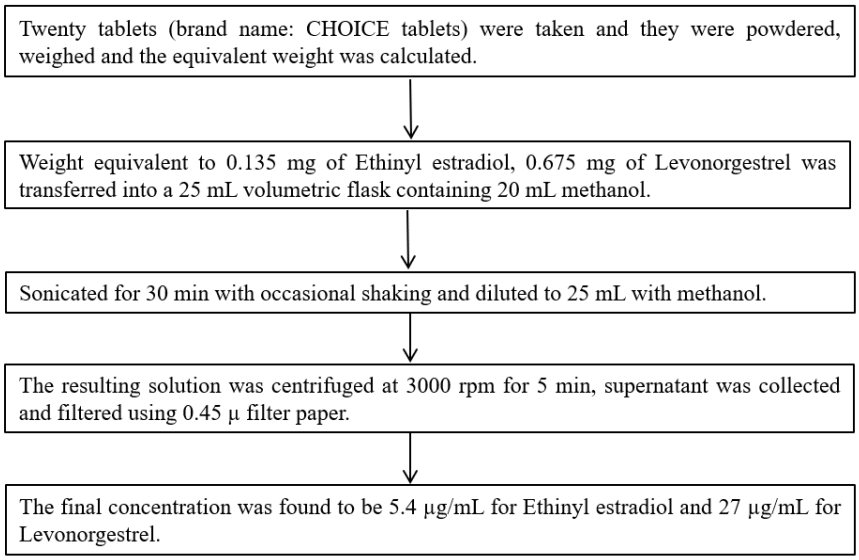
Accuracy

In order to test the method's accuracy, it was applied to CHOICE tablets to which addition of known amounts of Ethinyl estradiol, Levonorgestrel and Ferrous fumarate (bulk drug) in the proportion of 80%, 100%, and 120% of the label claim was added. This powder was then extracted and the results were detected by running a chromatogram in an optimized solvent system.

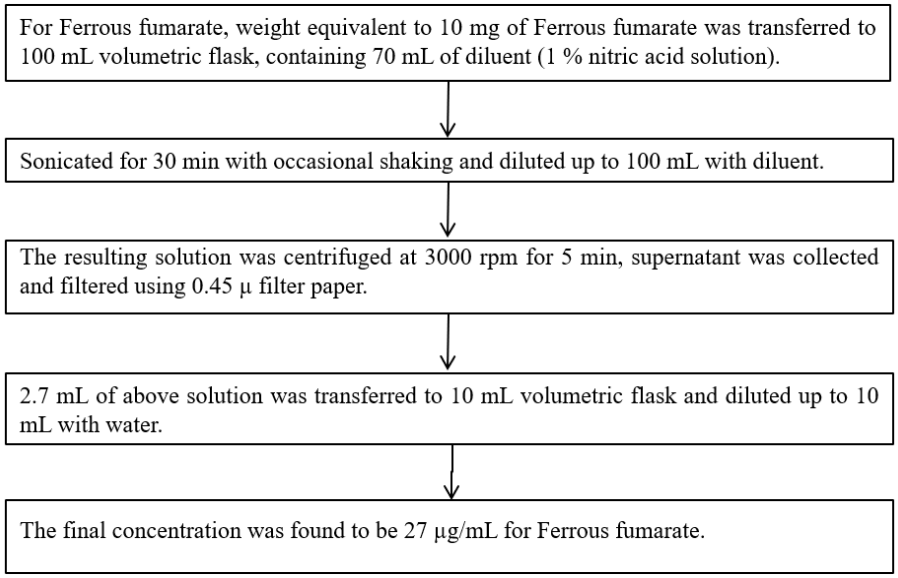
Formulation Analysis

Assay solutions of Ethinyl estradiol and Levonorgestrel were prepared in methanol, as these drugs are highly soluble in it. Whereas, Ferrous fumarate was prepared in 1% nitric acid as the drug is insoluble in solvents like water, methanol, acetonitrile and DMSO.

Assay 1



Assay 2



Results and Discussion

System suitability

Five standard solutions containing 5 µg/mL of Ethinyl estradiol, 25 µg/mL of Levonorgestrel and 25 µg/mL of ferrous fumarate were introduced into the HPLC system to determine its system suitability which is shown in Table 1.

Table 1 System suitability

Parameter	Ethinyl estradiol	Levonorgestrel	Ferrous fumarate	Formula	Limit
Retention time (Min)	6.66	9.87	2.86	--------	1<K<20
Tailing Factor	1.430	1.396	2.197	Tf= $W_{0.05\%}$/2f	Tf < 2
Number of theoretical plates (N)	6281	7955	2517	N=16(t/W)	N>2000

Linearity

Ethinyl estradiol, Levonorgestrel and Ferrous fumarate showed good correlation coefficient. The values of the correlation coefficient were 0.9997 for Ethinyl estradiol, 0.9992 for Levonorgestrel and 0.9995 for Ferrous fumarate, respectively.

Precision

Intraday and intermediate precision studies are represented in **Table 2**. The RSD values for repeatability and intermediate precision studies for the developed method were found to be <2% and were found to be in the alignment with the recommendations outlined by the ICH guidelines.

Precision (Repeatability and Intermediate precision)

Concentration (µg/mL)	Intraday (n= 6)			Interday (n= 6)		
	Concentration found ± standard deviation	% RSD	% drug Recovered	Concentration found ± Standard deviation	% RSD	% drug Recovered
Ethinyl estradiol						
5	5.02 ± 179.6	0.43	100.42	4.992 ± 455.5	1.12	99.84
20	19.94 ± 1106.1	0.73	99.71	20.05 ± 2053.6	1.34	100.27
40	39.83 ± 3006.5	1.00	99.58	39.62 ± 3891.9	1.98	99.05
Levonorgestrel						
25	24.59 ± 1680.8	0.50	98.37	24.58 ± 1550.8	0.20	98.35
100	98.48 ± 20736.6	1.52	98.48	100.59 ± 2523.6	0.32	100.59
200	200.32 ± 28144.2	0.98	100.16	201.52 ± 25199.6	0.80	100.76
Ferrous fumarate						
25	25.03 ± 3456.6	1.62	100.14	25.03 ± 2008.8	1.08	100.14
100	101.09 ± 4248.9	0.562	101.09	100.82 ± 4258.5	0.56	100.82
200	202.3 ± 5276.5	0.359	101.15	196.9 ± 5296.5	0.35	98.49

LOD and LOQ

The values found for LOD and LOQ are as per **Table 3**.

Limit of Detection and Limit of Quantitation

Parameter	Ethinyl estradiol (µg/mL)	Levonorgestrel (µg/mL)	Ferrous fumarate (µg/mL)
LOD	1.26	2.45	2.15
LOQ	3.62	4.54	4.85

Robustness

Wavelength and amount of mobile phase was altered at three levels i.e. -1, 0 and 1 and composition of mobile phase was varied at levels -2, 0 and 2, to calculate the impact on retention time and relative standard deviation. Insignificant variations in retention time and area under the curve was seen which is shown in Table 4, 5 and 6.

Robustness testing for Ethinyl estradiol. **Table 4**

Variable	Level	Retention time (min)	% RSD
Flow rate (mL/min)			
0.6	-1	7.35	0.65
0.7	0	7.12	0.24
0.8	+1	7.02	0.94
% of acetonitrile in mobile phase (mL)			
68	-2	7.96	1.02
70	0	7.89	0.94
72	+2	7.76	1.32
Wavelength (nm)			
264	-1	7.11	0.78
265	0	7.16	0.25
266	+1	7.20	1.06

Robustness testing for Levonorgestrel **Table 5**

Variable	Level	Retention time (min)	% RSD
Flow rate (mL/min)			
0.6	-1	11.44	1.02
0.7	0	11.32	0.85
0.8	+1	11.24	0.64
% of acetonitrile in mobile phase (mL)			
68	-2	11.22	1.00
70	0	11.32	0.96
72	+2	11.41	0.74
Wavelength (nm)			
264	-1	11.15	0.45
265	0	11.32	0.64
266	+1	11.42	0.84

Robustness testing for ferrous fumarate **Table 6**

Variable	Level	Retention time (min)	% RSD
Flow rate (mL/min)			
0.6	-1	2.68	0.45
0.7	0	2.57	0.25
0.8	+1	2.44	0.67
% of acetonitrile in mobile phase (mL)			
68	-2	2.64	1.04
70	0	2.57	0.86
72	+2	2.51	0.65
Wavelength (nm)			
264	-1	2.43	0.54
265	0	2.57	0.62
266	+1	2.76	0.97

Specificity

The method was found to be specific after analyzing the spectra. No hindrance of excipients was found and the peak purity of all three drugs was found to be more than 0.999.

Accuracy

The data presented in Table 7 clearly demonstrate favorable recovery results for Ethinyl estradiol, Levonorgestrel and Ferrous fumarate. Recoveries falling within the range of 98 to 102% were achieved across different concentration levels, further affirming the accuracy and reliability of the analytical procedure. Main findings from accuracy studies gave data in Table 7.

Accuracy studies **Table 7**

Tablet claimed content (mg/tablet)	Concentration added (mg)	Total concentration (mg)	Concentration found (mg) ± % RSD	Recovered %
Ethinyl estradiol				
0.03	0.024 (80%)	0.054	0.053 ± 0.19	99.89
0.03	0.03 (100%)	0.060	0.059 ± 0.42	99.42
0.03	0.036 (120%)	0.066	0.065 ± 0.44	99.43
Levonorgestrel				
0.15	0.12 (80%)	0.270	0.268 ± 0.09	99.45
0.15	0.15 (100%)	0.300	0.300 ± 0.61	100.08
0.15	0.18 (120%)	0.330	0.330 ± 0.44	100.03
Ferrous fumarate				
60	48 (80%)	108.00	107.87 ± 1.04	99.88
60	60 (100%)	120.00	120.38 ± 0.90	100.32
60	72 (120%)	132.00	131.98 ± 0.94	99.99

Analysis of a formulation

The amounts of Ethinyl estradiol, Levonorgestrel and Ferrous fumarate measured experimentally and reported as a percentage of the label claims were in good agreement and found to be between limits which is shown in Table 8.

Formulation analysis. **Table-8**

API and their strength in tablet	Mean ± SD (n= 6)	Recovery (%)
Ethinyl estradiol (0.03 mg)	0.029 ± 0.72	99.53
Levonorgestrel (0.15 mg)	0.149 ± 0.86	99.39
Ferrous fumarate (60 mg)	59.87 ± 0.34	99.79

Discussion

The applications of HPLC to pharmaceutical analysis are a significant improvement in terms of quality control. ICH guidelines were followed in the development and validation of the HPLC technique. UV detection allowed an accurate quantitation of steroidal compounds. In order to test the applicability of the analytical method, a thorough analysis of the procedure's linearity, accuracy, precision, and robustness was conducted on HPLC. The methodology was also effectively used to analyse tablets that were readily available on the market.

Conclusion

It was determined that the approach demonstrates linearity and selectivity within the concentration ranges of 25–250 µg/mL for both Levonorgestrel and Ferrous fumarate and 5–50 µg/mL for Ethinyl estradiol. The developed method provides better resolution between Ethinyl estradiol and Levonorgestrel with high specificity and good analytical time. All of these make the suggested method suitable for routine analysis of Ethinyl estradiol, Levonorgestrel and Ferrous fumarate. The method can be used to analyse Ethinyl estradiol, Levonorgestrel, and Ferrous fumarate from bulk drug and pharmaceutical tablet formulations with no hindrance of excipients. It can also be used to estimate the levels of Ethinyl estradiol, Levonorgestrel, and Ferrous fumarate in plasma and other biological fluids, as well as to analyse the kinetics of their degradation.

Acknowledgement

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Declaration of Interest: No conflict of interest found.

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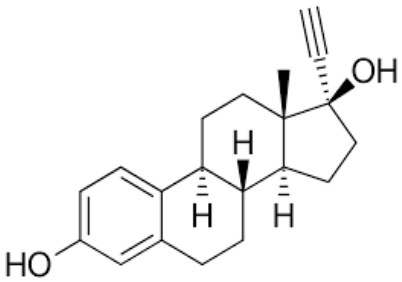

Fig. 1: Structure of Ethinyl estradiol

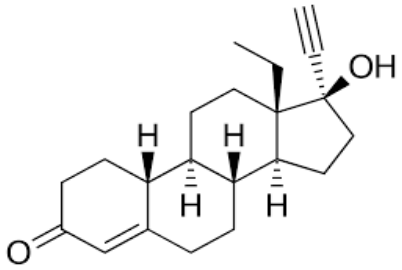

Fig. 2: Structure of Levonorgestrel

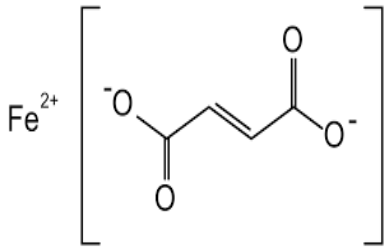

Fig. 3: Structure of Ferrous fumarate

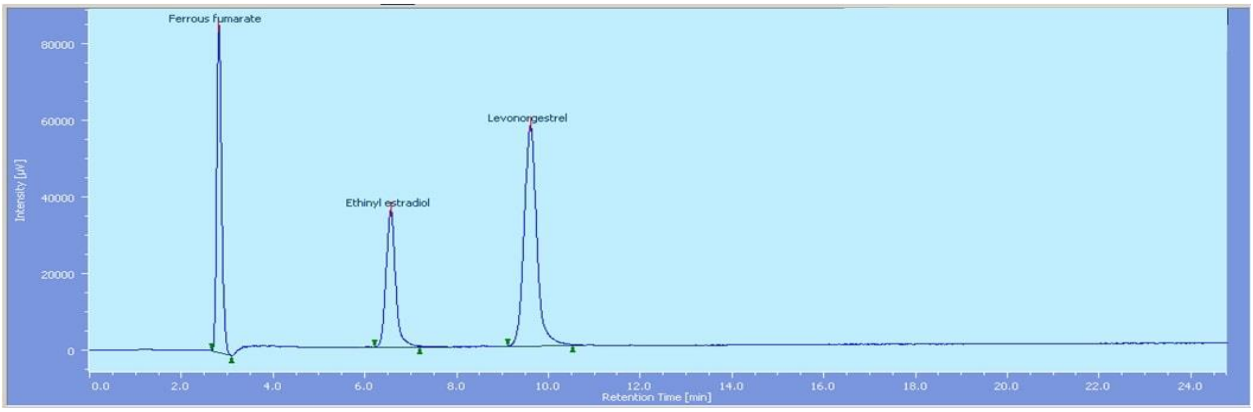

Fig. 4: Peaks of Ferrous fumarate, Ethinyl estradiol and Levonorgestrel respectively in mobile phase containing acetonitrile and water in 70: 30 ratio.

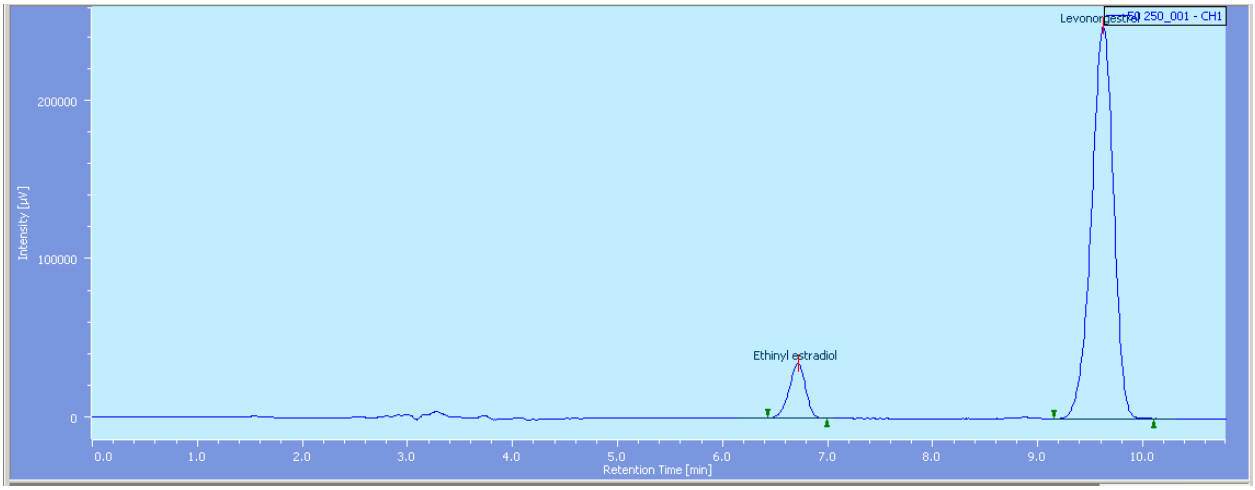

Fig. 5: Peak showing Ethinyl estradiol and Levonorgestrel response respectively in mobile phase containing acetonitrile and water in 70: 30 ratio

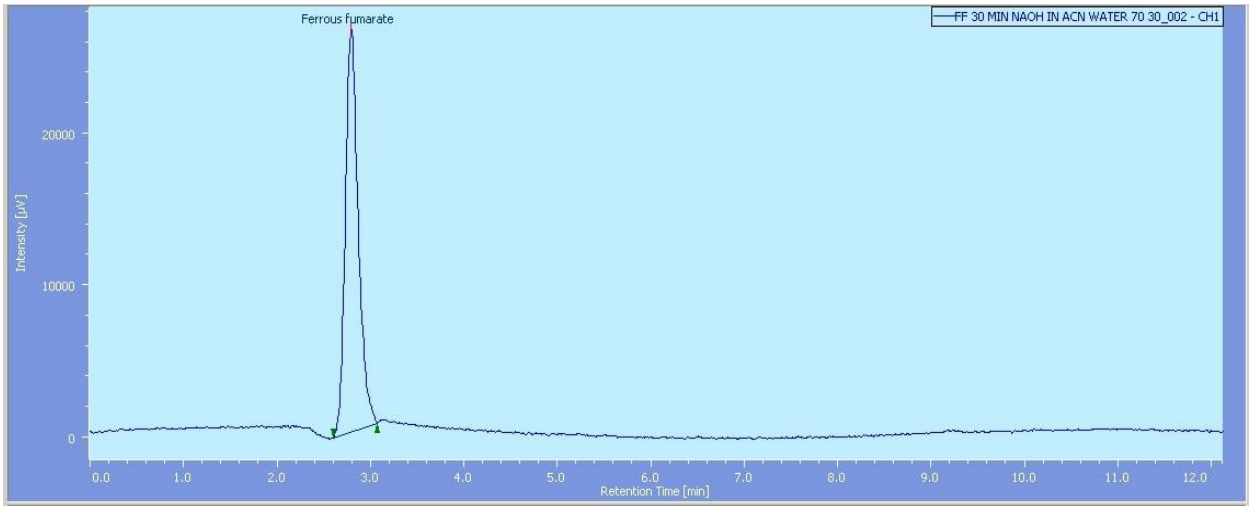

Fig. 6: Peak showing Ferrous fumarate response in mobile phase containing acetonitrile and water in 70: 30 ratio.

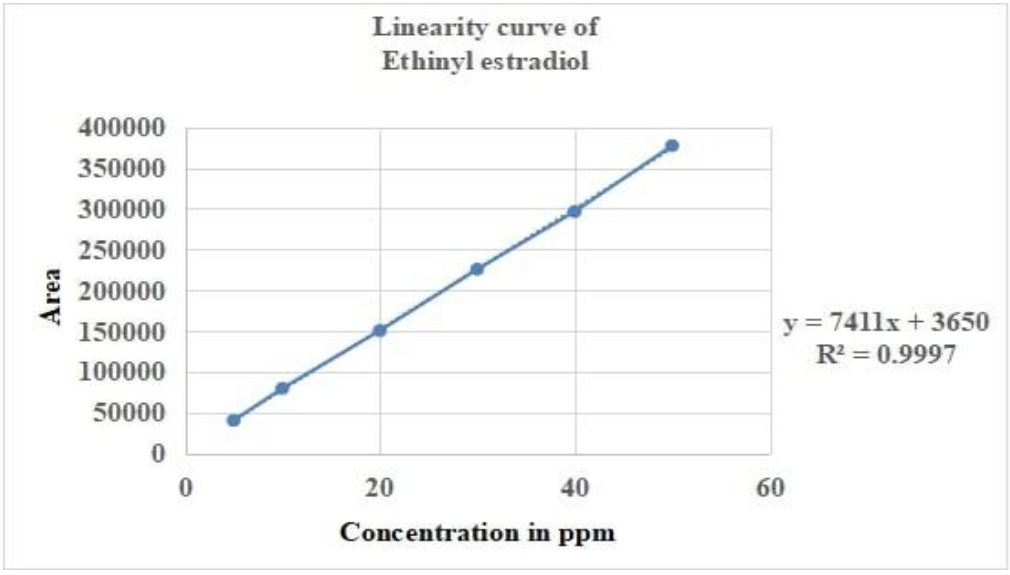

Figure 7: Linearity graph of Ethinyl esytradiol

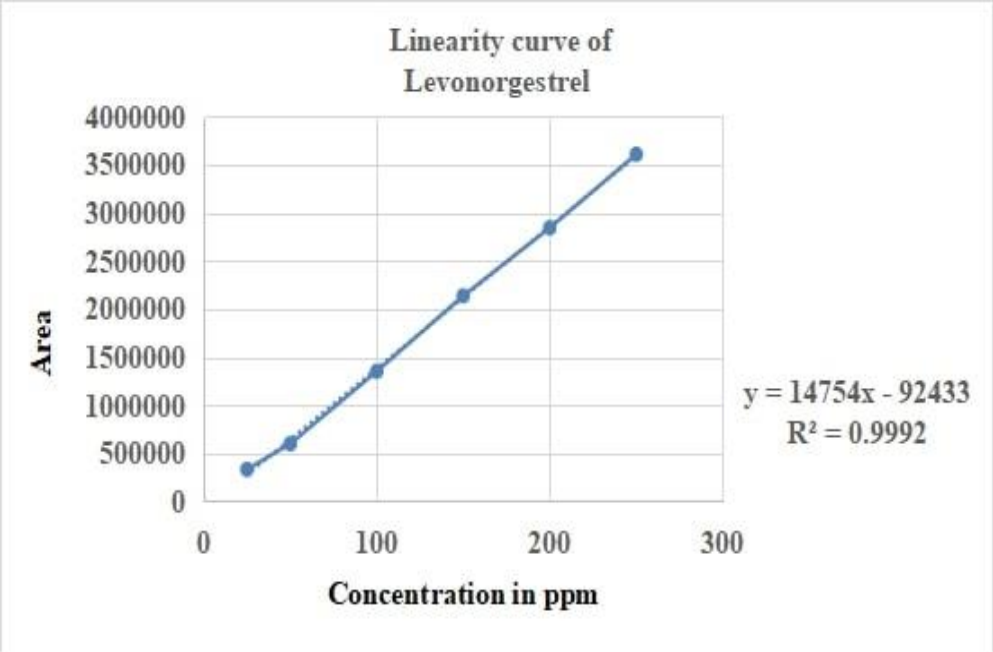

Figure 8: Linearity graph of Levonorgestrel

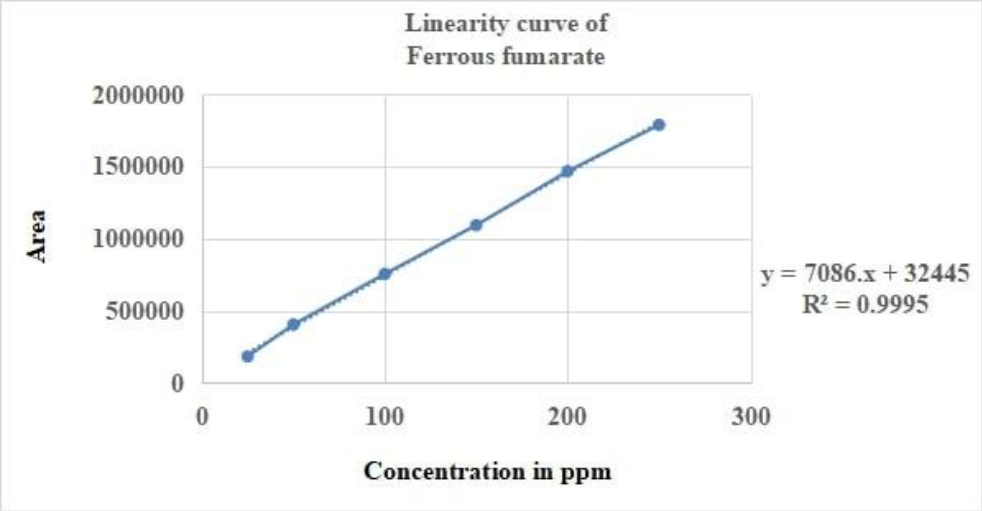

Figure 9: Linearity study of ferrous fumarate