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BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ETHINYL ESTRADIOL WITH ETHINYL ESTRADIOL-D4 AS INTERNAL STANDARD IN HUMAN K_2-EDTA PLASMA BY LC-MS/MS

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Keywords:

Ethinyl Estradiol, LC–MS/MS, Human plasma, Validation, Bioavailability

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ABSTRACT: A sensitive method was developed to determine Ethinyl Estradiol in human plasma by liquid chromatography-tandem mass spectrometric (LC–MS/MS). Separation of analyte and the Internal Standard (IS) Ethinyl Estradiol-d4 using SPE followed by LLE in plasma with TBME, was performed on a LC/MS/MS-API-5500 instrument with SB C18 HT (50mm×3.0mm) 1.8 µm columns at a flow rate of 0.300 ml/min with mobile phase consisting of Acetonitrile: 2 mM Ammonium Formate Buffer (80:20 v/v). The interface used with the API 5500 LC/MS/MS was used a turbo ion spray. The positive ions were measured in MRM mode for the analyte and IS. The method was linear over range of 5.000 - 308.560 pg/mL. The correlation coefficient is ≥ 0.9942 for Ethinyl Estradiol. The mean % recovery of extraction across QC of Ethinyl Estradiol in plasma was 68.48%. The reproducibility of Ethinyl Estradiol QC sample at room temperature and at 5 ºC was between 91.80 to 101.20% and 90.63 to 101.44% respectively. The % CV of intra and inter day precision were less than 19.74%. In the present study, a novel, fast, sensitive and robust method to quantify at very low concentration (pg/mL) Ethinyl Estradiol in human plasma using Ethinyl Estradiol-d4 as the IS was described.

INTRODUCTION: Hormonal therapy constitutes one of the treatment options available to women with acne. Ethinyl Estradiol is the most established and has well-documented efficacy in the treatment of acne. Ethinyl Estradiol is also known as Ethynyl Estradiol (EE) which is a derivative of 17α – Estradiol. It is the first orally active semi synthetic steroidal [1] Estrogen. Ethinyl Estradiol [2] is an orally bioactive Estrogen used in almost all modern formulations of combined oral contraceptive pills. Its Molecular formula is $C_{18}H_{24}O_2$ and Mol. mass is 296.40 g/mol.

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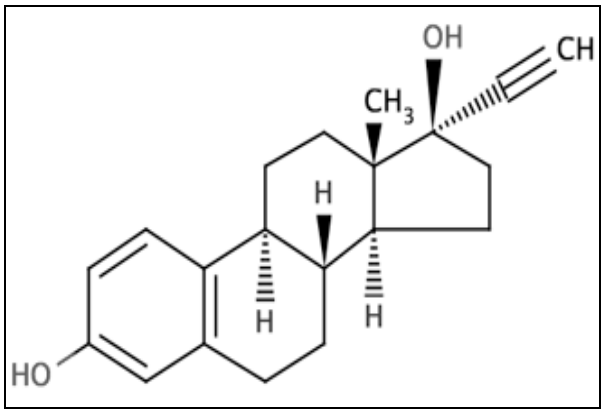
Chemically it is 17-Ethynyl-13-methyl-7, 8, 9, 11, 12, 14, 15, 16, 17-octahydro- 6H-cyclopenta[a]phenanthrene-3, 17-diol. As a lipophilic hormone, it diffuses readily through cellular membranes to bind to estrogen receptors situated in the nucleus. Ethinyl Estradiol [3-5] is absorbed with maximum plasma concentrations occurring within 2 hours after drug administration.

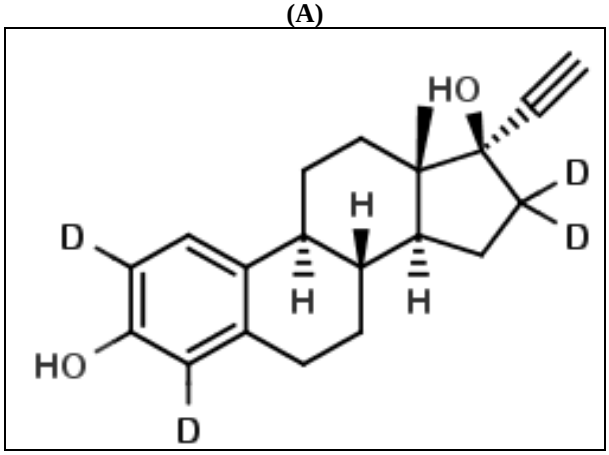
Ethinyl Estradiol is rapidly absorbed from the gastrointestinal tract but, due to first-pass metabolism in gut mucosa and liver, the bioavailability of Ethinyl Estradiol is approximately 43%. Ethinyl Estradiol is about 95 - 97% plasma protein bound, mainly to albumin. Ethinyl Estradiol is excreted in the urine and feces as glucuronide and sulfate conjugates, and it undergoes enter hepatic recirculation. The terminal elimination half-life of Ethinyl Estradiol after a

single dose was found to be about 18 hours. Ethinyl Estradiol-d4 is used as internal standard. Its Molecular formula is $C_{18}H_{20}D_4O_2$ and mol. mass is 276.41 g/mol. It is chemically 17α-Ethinyl Estradiol-2, 4, 16, 16-d4.

Literature surveys of the drug shows that HPLC [6-10] LC-MS [11-19] and GC-MS [20] methods for the determination of Ethinyl Estradiol in human plasma and in pharmaceutical formulations either as a single and in combination with other drugs. The various published method of analysis is available but the proposed method is optimized to quantify at very low concentration (pg/mL), and suitable for the various laboratory conditions **(Fig. 1)**.



(A)



(B)

FIG. 1: STRUCTURE OF (A) ETHINYL ESTRADIOL AND (B) ETHINYL ESTRADIOL-D4

Experimental Requirement:

Chemicals and Reagents: The Analyte Ethinyl Estradiol (99.40%) was obtained from Famy care limited, Gujarat. Internal standard Ethinyl Estradiol-d4 (98.30%) was obtained from CDN isotopes, Canada. All other solvents used were of analytical grade only. Formic acid, Acetone, Sodium carbonate, Acetonitrile and Methanol are obtained from Merck Chemicals, Mumbai. Ammonium Formate and Dansyl Chloride are obtained from Fluka Pvt. Ltd. Ter-Butyl Methyl ether was obtained from Spectrochem, Mumbai. Human K-EDTA Plasma aliquot was obtained from blood bank.

Instruments: Shimadzu LC-20AD series HPLC was used. All Weighing were done on Micro Balance model ME5/SE-2 Instrument manufactured by Sartorius. Micro-Pipettes manufactured by brand were used. Ultrasonic bath of Pharmatek Scientifics was used.

Chromatographic Conditions: LC-20AD series HPLC from Shimadzu technologies was used. It consists an auto sampler (Shimadzu SIL-HTc), LC-20AD serial pumps, manual injector, DGU-$20A_3$ prominence degasser, analytical column SB C18 HT (50mm×3.0mm) 1.8 µm (Agilent) with flow rate of 0.300 ml/min and mobile phase consisting of Acetonitrile: 2 mM Ammonium Formate Buffer (80:20 v/v). Data acquisition system and quantization program (Applied biosystems analyst® software version1.5) was used for the determination of Ethinyl Estradiol in human plasma.

Mass Spectrometric Conditions: LC-MS/MS analysis was performed on an API 5500 LC/MS/MS (Applied biosystems) triple quadrupole mass spectrometer equipped with an electro spray ionization (ESI) interface with turbo ion spray. The positive ions were measured in MRM mode for the analyte and Internal Standard. For operation in MS/MS mode, a mass spectrometer fitted with an electrospray ion source interface was used for analysis. The source was operated initially at a temperature of 400 ^{0}C. The mass spectrometer was programmed to monitor the protonated molecule $[M+H]^+$ at m/z 530.30 via the first quadrupole filter (Q1), the product ion at m/z 171.10 was monitored via the third quadrupole filter (Q3). Finally, all MS parameters were manually fine tuned to obtain the highest MRM signals. The MRM transition m/z 530.30/171.10 was monitored for the detection of Ethinyl Estradiol as shown in **(Fig. 2)**. In the same way for internal standard also processed as shown in **(Fig. 3).**

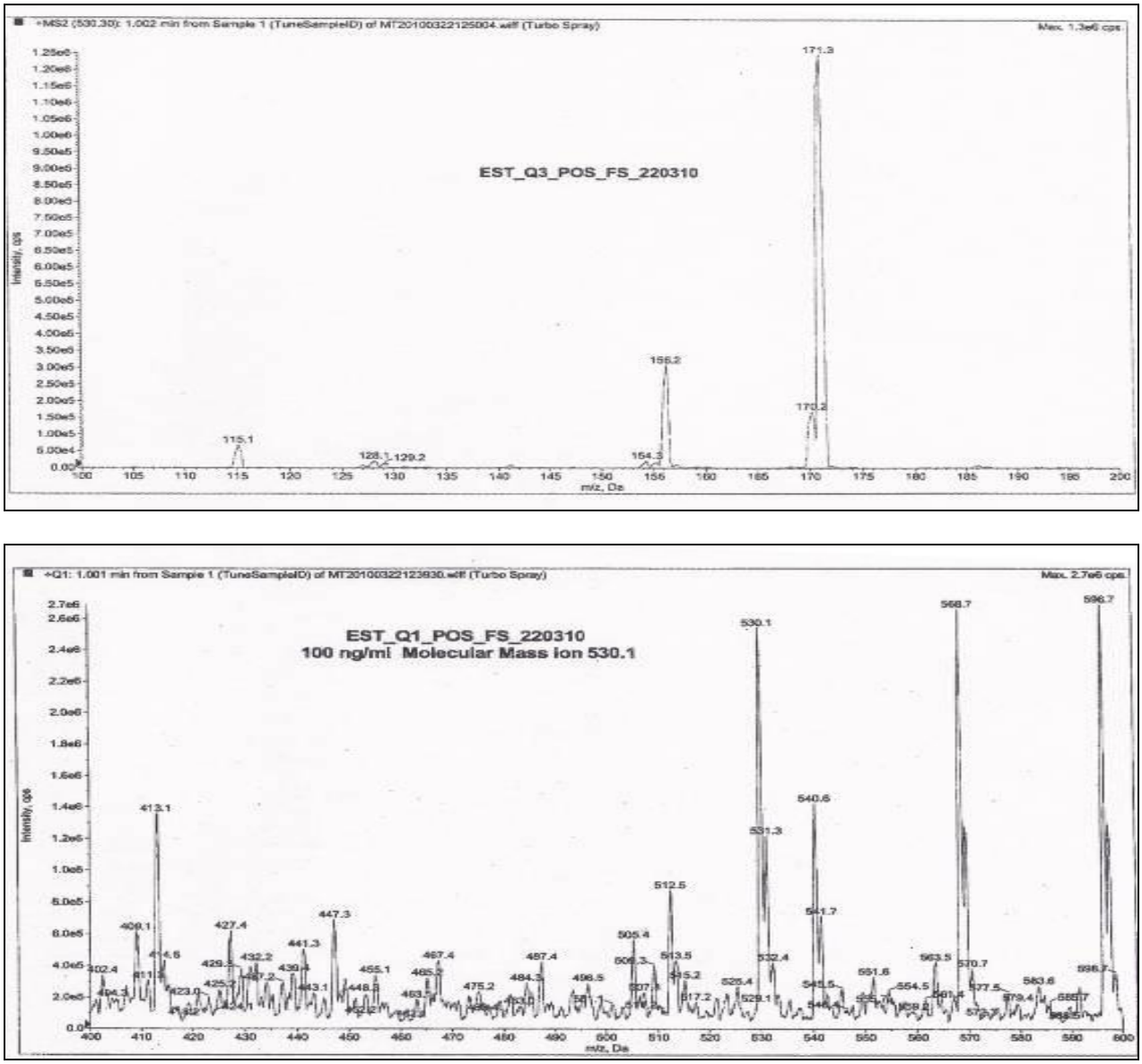
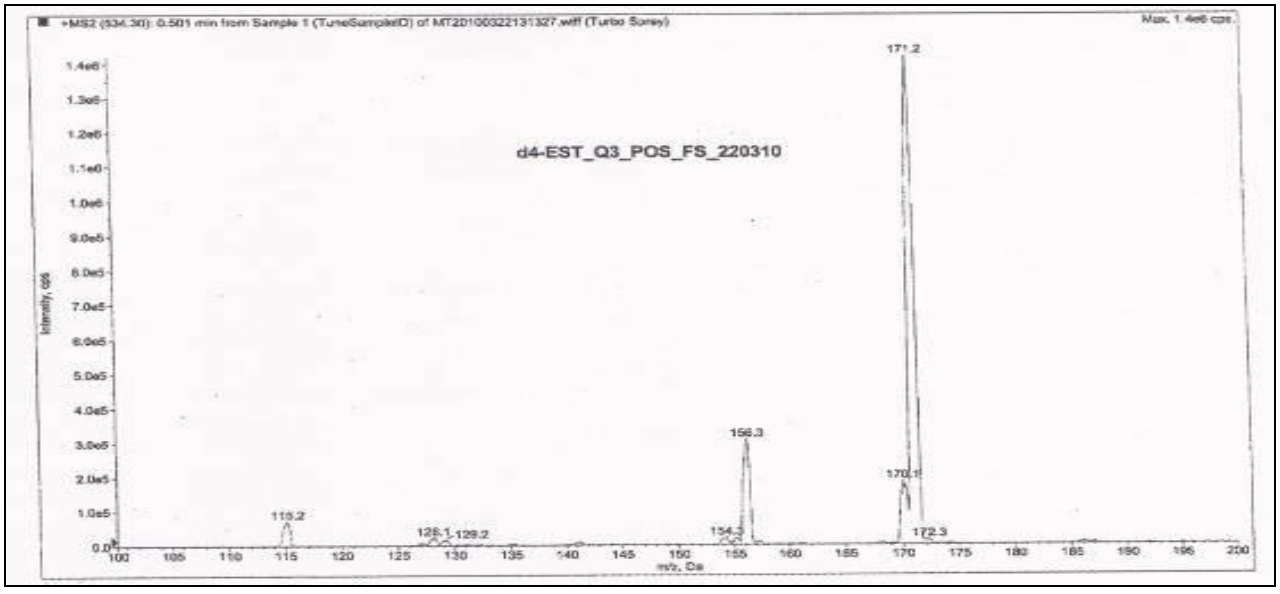


FIG. 2: THE MRM TRANSITION M/Z 530.30/171.10 DETECTION OF ETHINYL ESTRADIOL



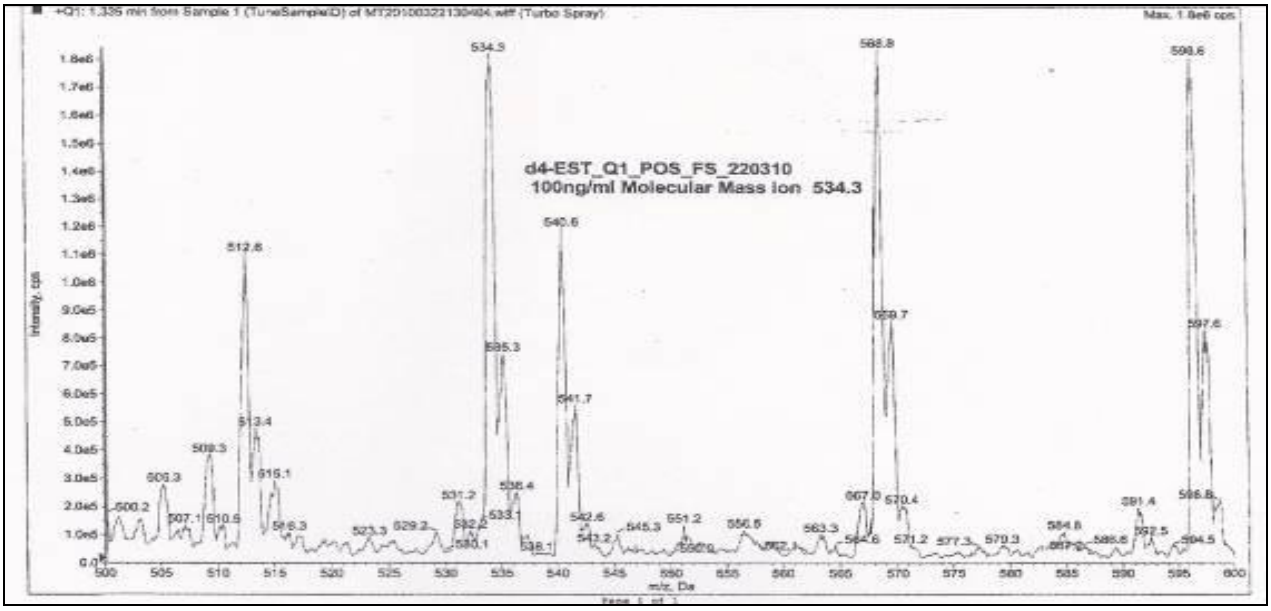


FIG. 3: THE MRM TRANSITION M/Z 530.30/171.10 DETECTION OF ETHINYL ESTRADIOL- D4 (IS)

Experimental Procedure:

Preparations of Calibration Standard and Samples: Stock solution of Ethinyl Estradiol (400.00µg/mL) was prepared by dissolving 2.0 mg in 2 mL of 100% v/v Methanol and made up to 5 mL with same.

The Internal Standard stock solution (400.00µg/mL) was prepared by dissolving Ethinyl Estradiol-d4 2 mg in 2 mL of 100% v/v Methanol and made up to 5 mL with same.

Standard Intermediate solution of Ethinyl Estradiol (1.200 µg/mL) was prepared by transferring 15 µL stock solution to a 5 mL volumetric flask containing in 2 mL 20 % v/v Methanol and made up to volume 5 mL.

Standard Intermediate solution of Ethinyl Estradiol-d4 (4.000 µg/mL) was prepared by transferring 100 µL internal standard stock solution to a 10 mL volumetric flask containing in 5 mL 20 % v/v Methanol and made up to volume 10 mL.

Internal standard working solution of 10.000 ng/mL was prepared by transferring 250 µL internal standard stock solution (4.000 µg/mL) to a 100 mL volumetric flask containing 50 mL 20 % v/v Methanol and made up to volume 100 mL.

Working standard solutions for the plasma calibration curve and QC samples were prepared by diluting stock of Ethinyl Estradiol with 20 % v/v Methanol to yield the required concentrations.

Calibration Curve Standards: To prepare the calibration curve samples, working standard solutions (20 µL) was taken from Ethinyl Estradiol and added to 980 µL of blank plasma to achieve the calibration curve standard concentrations. One calibration standard curve at concentrations of 5.000, 10.000, 20.300, 60.900, 121.800, 182.700, 243.600 and 308.560 pg/mL for Ethinyl Estradiol and two blanks with internal standard and two blanks without internal standard were analyzed. The QC samples were prepared in bulk by mixing 0.2 parts of the respective working QC solutions each of Ethinyl Estradiol with 9.8 parts of blank human plasma [9]. Six sets of QC samples at concentrations of 5.200 pg/mL, 15.600 pg/mL, 44.880 pg/mL, 89.760 pg/mL and 179.520 pg/mL concentrations for Ethinyl Estradiol were assayed [10-11] with mobile phase consisting of Acetonitrile: 2 mM Ammonium Formate Buffer (80:20 v/v), with each batch run of the validation.

Method Validation: The method was validated in accordance with ICH Guidelines. The objective of the work is to validate specific LC/MS/MS method for the determination of Ethinyl Estradiol in human plasma to ensure the selectivity, accuracy, reproducibility and sensitivity of the method.

RESULTS AND DISCUSSION:

Calibration Curve Standards: Calibration curve was found to be consistently accurate and precise for Ethinyl Estradiol over 5.000-308.560 pg/mL as shown in (**Fig. 4)**. The retention times of Ethinyl

Estradiol and Ethinyl Estradiol-d4 are 3.42 min ± 0.30 min and 3.45 min ± 0.30 min respectively. The overall chromatographic run time is 4.0 min. The correlation coefficient is 0.9942 for Ethinyl Estradiol. Back calculations were made from the calibration curve to determine Ethinyl Estradiol concentrations of each calibration standard as shown in **(Fig. 5).**

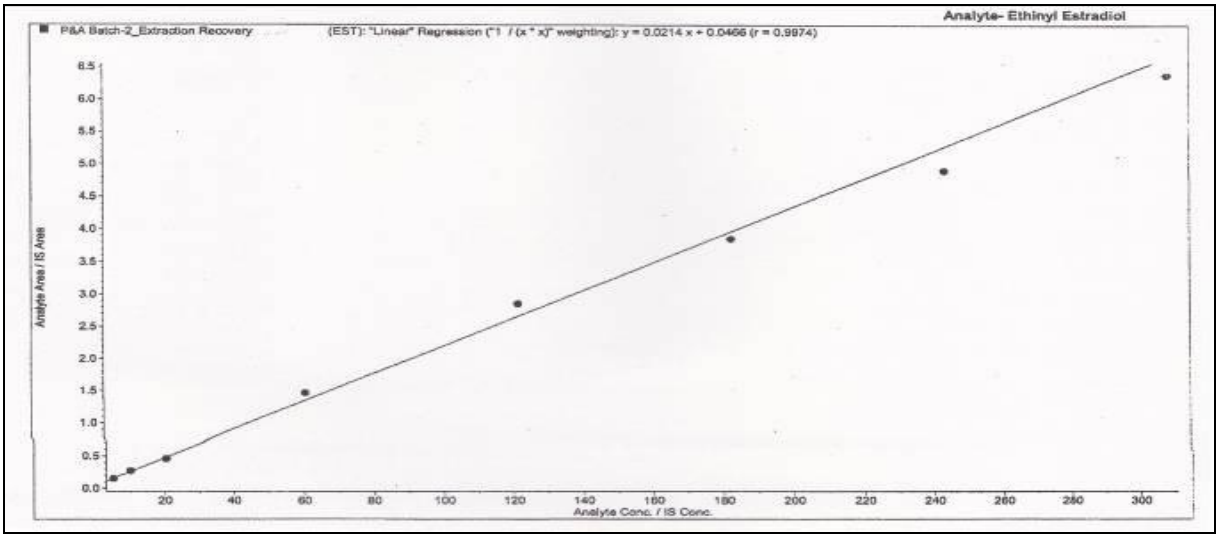


FIG. 4: CALIBRATION CURVE OF ETHINYL ESTRADIOL

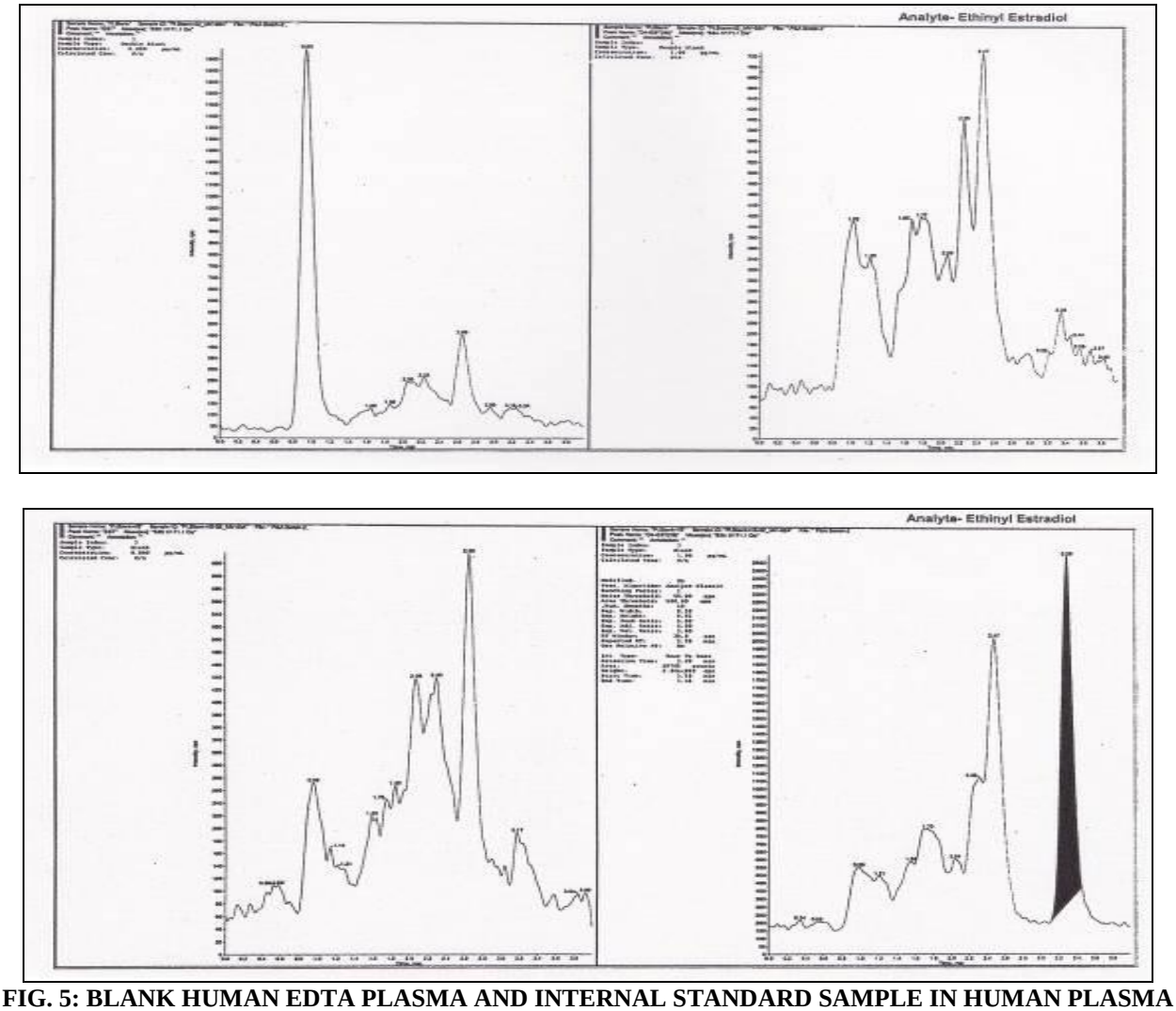


FIG. 5: BLANK HUMAN EDTA PLASMA AND INTERNAL STANDARD SAMPLE IN HUMAN PLASMA

Extraction Recovery: The percentage recovery of Ethinyl Estradiol was determined by comparing the mean peak area of Ethinyl Estradiol samples with freshly prepared un-extracted and extracted samples respectively. The % recovery of Ethinyl Estradiol is 66.64%. The Mean recovery of Ethinyl Estradiol across QC levels is 68.48 as shown in **(Table 1).**

TABLE 1: EXTRACTION RECOVERY OF ETHINYL ESTRADIOL IN HUMAN PLASMA (n = 6)

Peak Response (Mean ± SD)		% CV	% Recovery	Mean Recovery of Across QC
Un extracted	Extracted			
17434 ± 670	-	3.84	66.64	68.48 ± 2.29
-	11618 ± 908	7.82		

Dilution Integrity: Dilution integrity was carried out at six replicate two times diluted and four times diluted were prepared and concentrations were calculated against the freshly prepared calibration curve. The % accuracy of Ethinyl Estradiol nominal concentrations were 100.82% and 101.13% for 1 in 2 dilutions and 1 in 4 dilutions respectively. The % CV is 0.77% to 2.25%.

Reproducibility: Whole batch reinjection reproducibility experiment, samples were kept at room temperature for 15 hrs 52 min after the initial analysis and were re-injected. The 5 sets of QC sample of Concentrations (5.200, 15.600, 44.880, 89.760, and 179.520 pg/mL) were calculated to determine precision and accuracy after reinjection. The accuracy of Ethinyl Estradiol QC samples in reinjection was between 91.80 to 101.20%. The Precision (%CV) of Ethinyl Estradiol QC samples in reinjection was between 1.40 to 6.44%. The partial reinjection reproducibility experiment, samples were kept in the auto sampler at 5 ^{0}C for 18 hrs 10 min and re-injected. The 5 sets of QC sample of Concentrations (5.200, 15.600, 44.880, 89.760, and 179.520 pg/mL) were calculated to determine Precision and accuracy after injection. The Accuracy of Ethinyl Estradiol QC samples in reinjection was between 90.63 to 101.44%. The Precision (%CV) of Ethinyl Estradiol QC samples in reinjection was between 1.49 to 9.40%.

Ruggedness: To evaluate ruggedness experiment with different column (Column 1 and Column 2), samples were re-injected on different columns with same and specifications, The 5 sets of QC sample of Concentrations (5.200, 15.600, 44.880, 89.760, and 179.520 pg/mL) were calculated to determine precision and accuracy. The accuracy of Ethinyl Estradiol QC samples within the range of 95.00 to 104.79 %. The Precision of Ethinyl Estradiol QC samples within the range of 1.64 to 6.41%.

To evaluate ruggedness experiment with different analysts, one batch was processed by different analyst. The run consisted of a calibration curve standards and 6 replicate of 5 sets of QC sample Concentrations (5.200, 15.600, 44.880, 89.760, and 179.520 pg/mL). The accuracy of Ethinyl Estradiol QC samples within the range of 101.85 to 113.00%. The Precision of Ethinyl Estradiol QC samples within the range of 1.59 to 9.63% as shown in **(Table 2)**.

TABLE 2: SUMMERY OF RUGGEDNESS OF ETHINYL ESTRADIOL IN HUMAN PLASMA (n = 6)

Compound QC Concentration (pg/mL)	Column				Analyst		
	Concentration Found (Mean ± SD)		% CV	% Mean Recovery	Concentration Found (Mean ± SD)	% CV	% Mean Recovery
	Column 1	Column 2					
5.2	5.171 ± 0.62	5.728 ± 0.36	6.41	104.79	5.87 ± 0.56	9.63	113.00
15.6	15.07 ± 1.07	14.74 ± 0.93	6.31	95.56	16.26 ± 1.11	6.84	104.25
44.88	46.42 ± 1.18	45.77 ± 0.75	1.64	102.72	48.70 ± 1.09	2.24	108.52
89.76	91.16 ± 5.72	88.70 ± 4.09	4.62	100.19	94.87 ± 4.73	5.00	105.70
179.52	172.65 ±4.52	168.43 ± 7.17	4.26	95.00	182.84 ± 2.90	1.59	101.85

Precision and Accuracy: Inter batch accuracy and precision were assessed by analyzing 5 sets of calibration curves for Ethinyl Estradiol and 5 sets of QC samples, 6 replicates. The inter batch percentage of nominal concentrations (5.200, 15.600, 44.880, 89.760, and 179.520 pg/mL) for Ethinyl Estradiol was ranged between 98.43 to 103.15%. The Inter batch percentage of coefficient of variation for Ethinyl Estradiol was ranged between 4.62 to 19.74%. The Intra batch

percentage of nominal concentrations for Ethinyl Estradiol is 86.91 to 101.76 %. The Intra batch percentage of coefficient of variation for Ethinyl Estradiol is 1.58 to 5.21 as shown in **Table 3.**

TABLE 3: SUMMERY OF PRECISION AND ACCURACY OF ETHINYL ESTRADIOL IN HUMAN PLASMA

Compound QC Concentration (pg/mL) (n = 6)	Inter Day			Intra Day		
	Concentration Found (Mean ± SD)	% Recovery	% CV	Concentration Found (Mean ± SD)	% Recovery	% CV
5.2	5.119 ± 1.01	98.43	19.74	4.52 ± 0.49	86.91	5.21
15.6	15.74 ± 1.18	100.95	7.52	15.87 ± 0.26	101.76	1.61
44.88	45.96 ± 3.14	102.41	6.84	43.03 ± 1.52	95.87	3.54
89.76	92.58 ± 6.03	103.15	6.52	85.93 ± 2.58	95.74	3.01
179.52	178.18 ± 8.23	99.25	4.62	173.35 ± 2.74	96.56	1.58

Specificity and Selectivity: During specificity run, prepare the standard in one of the screened blank plasma including working range of internal standard. Blank plasma samples from 8 different lots, 6 standards were processed according to the extraction procedure. The responses for the blank plasma from 8 different lots were compared to the standard of the two analytes and internal standard. No significant response (≤ 20% for the analyte response and ≤5% of the internal standard response) was observed at the retention times of the analytes or the internal standard in blank plasma as compared to the standard. For selectivity standards of analyte and working range of internal standard separately on the recovery basis in 6 screened plasma blanks.

Stability Experiments: Bench Top Stability was done on QC sample deep freezer for 21 hrs 15 min, Dry ice stability for 45 hr 15 min, Post extracted refrigerator stability 2-8 ^{0}C for 29 hrs 50 min, Dry extract stability 2-8 °C for 29 hrs 33 min, Freeze - Thaw stability frozen at -70±15 ^{0}C & -20±5 ^{0}C, In-Injector stability for 49 hrs 52 min at 5 ^{0}C and were processed and analyzed. The mean % change, % CV and % recovery for QC samples storage condition of Ethinyl Estradiol were shown in **Table 4**.

TABLE 4: SUMMARY OF STABILITY EXPERIMENT OF ETHINYL ESTRADIOL IN HUMAN PLASMA UNDER VARIOUS CONDITIONS (n = 6)

Storage Conditions	Concentration (pg/mL)		% CV	% Recovery	% Change
	Added	Found			
Bench Top Stability (21 hrs 15 min)	179.520	166.343	2.76	92.66	2.26
Dry ice stability (45 hr 15 min)	179.520	168.399	1.97	93.80	3.52
Post Extracted Refrigerator Stability (29 hrs 50 min)	179.520	160.885	3.77	89.62	-0.67
Dry Extract stability (29 hrs 33 min)	179.520	165.004	4.55	91.91	1.87
Freeze - Thaw Stability (-70±15 ^{0}C)	179.520	160.412	3.34	89.36	-0.88
Freeze - Thaw Stability (-20±5 ^{0}C)	179.520	162.412	1.42	90.47	0.36
In-Injector Stability (49 hrs 52 min)	179.520	158.663	2.55	88.38	-2.04

CONCLUSION: The proposed method was found to be simple, sensitive, rapid and economical for the determination. All the results obtained are within the acceptance criteria. The developed method is suitable for estimation of plasma at low concentration (pg/mL) of Ethinyl Estradiol as a single analytical run. The high extraction efficiency, low limit of quantization, and wide linear dynamic range make this a suitable method for use in clinical samples for bioequivalence studies following oral administration of Ethinyl Estradiol (0.03 mg) tablets in healthy human subjects.

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