Highly Sensitive and Robust Quantification Method for Ethinyl Estradiol and Drospirenone in Plasma

SCIEX Triple Quad™ 5500 LC-MS/MS System and UPLC Chromatography

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Introduction

Estrogens and progesterone play an important role in fertility and sexual development, as well as in cancer risk. In modern world estrogen and progesterone combination based drugs are widely used as most effective contraceptive agents. One of such combination is Ethinyl estradiol (EE, Figure 2a) and Drospirenone. The mean bioavailability of EE is reported to be 45%. EE is known to undergo extensive metabolism and also can be highly protein bound makes its bio availability very low in human system. Drospirenone (Figure 2b) is a novel synthetic progestogen with a pharmacological profile similar to its natural progesterone. It will be metabolized completely but the metabolites are not shown any biological activities and undergo hepatic and renal elimination. Drospirenone and ethinyl estradiol combination in formulation have similar efficiency and safety profile to other low dose oral contraceptive. It has been also reported that it has less side effect with regards to weight gain, mood change etc. Highly sensitive and accurate low level quantitation becomes essential for the bioequivalence studies for such molecules. The main objective of this work is to develop and validate a highly sensitive and reproducible method for EE (1.0 pg/ml) in human plasma using SCIEX Triple Quad™ 5500 LC/MS/MS system (Fig 1).

Figure 1: SCIEX Triple Quad™ 5500 system

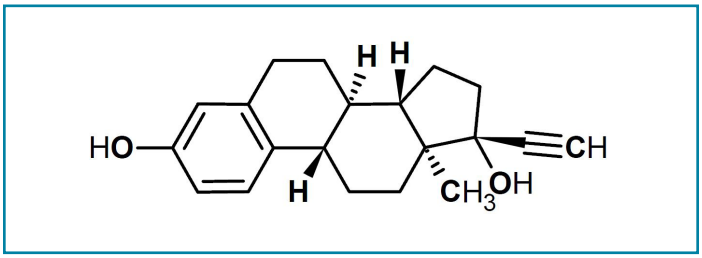

Figure 2a: Ethinyl Estradiol

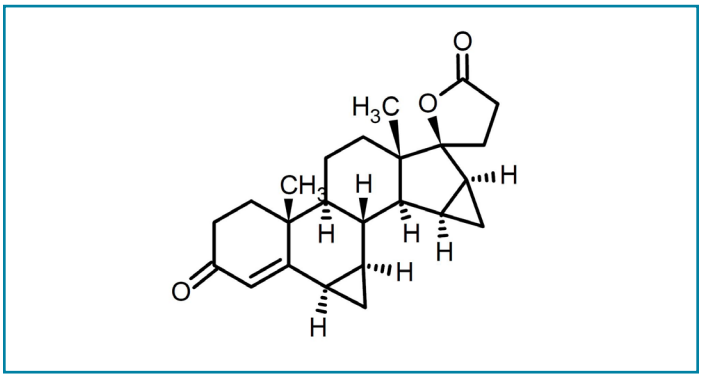

Figure 2b: Drospirenone

Key Features of the Method using SCIEX Triple Quad™ 5500 System

- **A sensitive, reproducible and cost effective LC/MS/MS method** was developed for a GLP bioanalytical laboratory using simple liquid-liquid extraction sample preparation method
- **The LLOQ for Ethinyl Estradiol in plasma was 0.993 pg/ml (10 fg on column)** S/N ratio of 68 with good precision and accuracy for n = 12 inj. in human plasma
- **Accuracy and precision** for Ethinyl Estradiol and Drospirenone are between 80-120% meeting the validation requirements for regulated bio analytical labs
- **New, patented QJet® 2 Ion Guide improves sensitivity and robustness:** The patented QJet® 2 Ion Guide design yields improved ion containment and operates at higher pressures providing better collisional focusing to enhance ion transmission for ultimate sensitivity
- **Next-generation eQ™ Electronics on** SCIEX Triple Quad™ 5500 provide improved performance at ultra-low MRM dwell times for improved support of fast LC and narrow peak widths.

Material and Methods

Sample Preparation

Plasma (500 µL) samples were spiked (2%) with Ethinyl Estradiol (EE) and Drospirenone standard with 10 µL internal standard solution. The samples were vortex with 2ml of TBME: n-Hexane mixture and centrifuged at 4^{0}C. Organic phase were collected and evaporated to dryness under nitrogen stream. The residue in each tube were dissolved in the 200 µl of Ammonium bicarbonate (pH:11) followed by 2min vortex. Dansyl chloride (0.5 mg/mL solution in acetone) was added to derivatized the Ethinyl Estradiol. Reaction mixture tubes were kept for 10 min at 60^{0}C in water bath. LLE was again performed with TBME: n-Hexane (2mL) followed by vortex and centrifugation. Organic phase were collected and evaporated and finally reconstituted in acetonitrile and water (200µL) for quantitation in SCIEX Triple Quad™ 5500 LC/MS/MS system

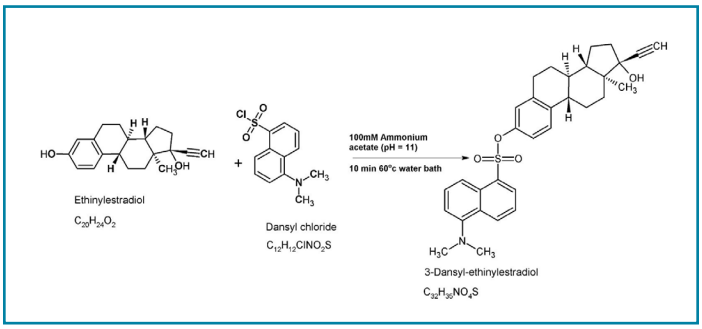

Figure 3: Chemical derivatization of Ethinyl estradiol using dansyl chloride

HPLC condition

A Shimadzu Nexera with 30AC auto sampler system was used, with a C18 (50 x 2.1 mm, 5 µm) analytical column maintained at 30^{0}C. A gradient elution was employed, consisting of 5mM Ammonium Formate buffer (mobile phase A) and acetonitrile:methanol (mobile phase B), at a flow rate of 300 µL/min. The total run-time for the method was 8.0 minutes. The injection volume was set to 10 µL. The rinsing solution was Methanol: Water (50/50) mixture.

Mass Spectrometry conditions

An SCIEX Triple Quad™ 5500 LC/MS/MS system equipped with Turbo V™ source was used, in positive Electrospray Ionization (ESI) mode. The optimized MRM for the analyte and internal standard are summarized in Table 1. The source parameters were optimized in flow injection analysis. Quadrupole mass analyzers (Q1 and Q3) were set at unit resolution for quantitative analysis. The mass spectrometer was operated in positive ionization mode with electrospray voltage +5500 V and source temperature of 550^{0}C. Nitrogen was used as nebulizing gas (GS1), drying gas (GS2) and curtain gas at 50, 55 and 30 arbitrary unit, respectively. Valco valve was used to divert the flow to avoid the interference during the chromatographic run.

Table 1: MRM transitions and optimised MS parameter for compounds

Analyte	MRM	Dwell Time (ms)	DP	EP	CE	CXP
Dansyl-Ethinyl Estradiol	530.2/171.1	200	100	15	49	16
D4 - Dansyl-Ethinyl Estradiol	534.2/171.0	200	100	15	49	16
Drospirenone	367.1/97.00	200	100	121	29	14
D4-Drospirenone	371.1/97.00	200	100	121	29	14

Results

Ethinyl estradiol was derivatised with dansyl chloride to obtain the maximum sensitivity in plasma sample (Fig 3). Dansyl - EE produced the Q1MS ion m/z 530.2 and major product ion 171.1 which correspond to 5-(dimethylamino)-naphthalene moiety. The best ionization was achieved in ESI positive mode. The mass spectrometric parameters for both the compounds are given in Table 1. A linear calibration curve was constructed using the $1/X^2$ regression. The calibration curve for EE was linear over a dynamic range of 0.993-300.48 pg/mL in plasma sample (Fig 8) with an r value 0.9980 and similarly, calibration curve for drospirenone shown in figure 9 has r value 0.9994

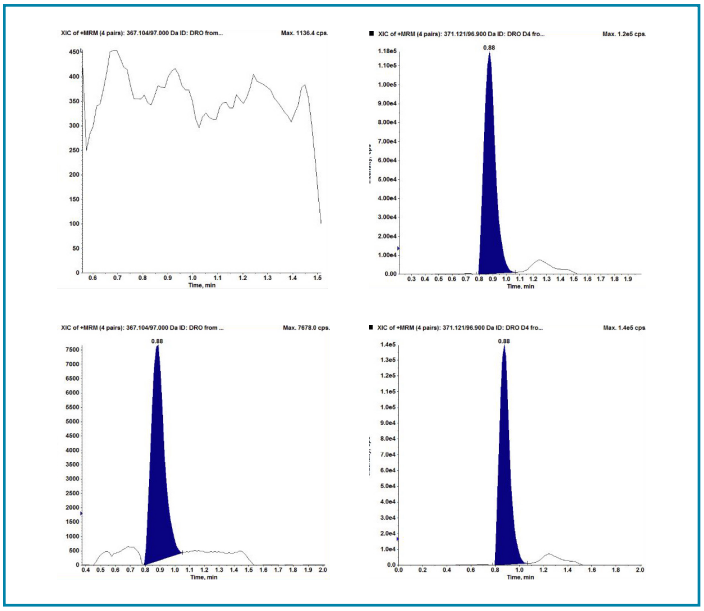

Figure 4: The chromatograms for drospirenone in zero blank plasma sample (upper pane) and 1003.63 pg/mL spiked in plasma sample (lower pane)

Several precision and accuracy batches were processed to evaluate the developed method. Results of EE and Drospirenone for precision and accuracy in batch-1 were given in Table 3 and 4. Different QCs level samples (n = 12) were also evaluated for precision and accuracy along with the calibration curve. Results were shown in Table 2. The EE and Drospirenone eluted at 0.88 min and 4.10 min with minimum background noise in an 8 min chromatographic run time (Fig 4 and Fig 6). The signal to noise ratio of EE at LOQ (0.993 pg/mL) in plasma is 68.3 calculated using sigma standard deviation of the baseline (Fig 7).

Table 2: Statistic calculation of quality controls (n=12 inj) in human plasma sample for Dansyl-Ethinyl Estradiol and Drospirenone

Analyte	Sample Name	Exp. Con (pg/ml)	Cal. Con (pg/ml)	Number of values used	%CV	Accuracy
Ethinyl Estradiol	LLOQ	0.9980	1.0406	12	4.5702	104.27
	LQC -1	2.6740	2.7786	12	1.6824	103.91
	MQC -1	150.2430	149.2491	12	1.0126	99.33
	HQC -1	246.3980	242.2303	12	2.2463	98.30
Drospirenone	LLOQ	1004.0070	1052.0814	12	2.0557	104.78
	LQC -1	2706.2190	2885.9109	12	0.8237	106.63
	MQC -1	84569.3450	84438.5864	12	1.0554	99.84
	HQC -1	140948.9090	141724.8942	12	2.464	100.55

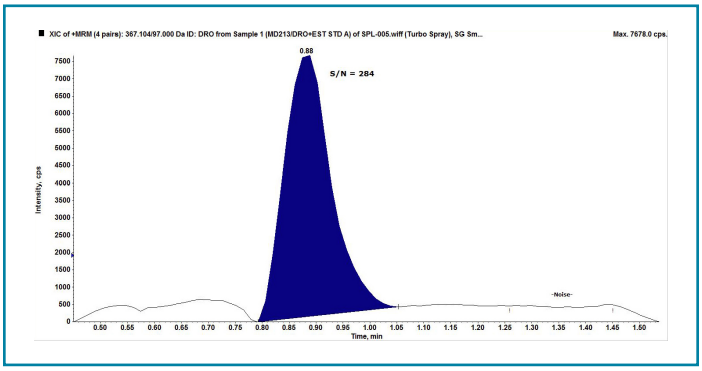

Figure 5: Signal to noise calculation for Drospirenone in extracted plasma sample at LLOQ level (1003.63 pg/mL) is 284

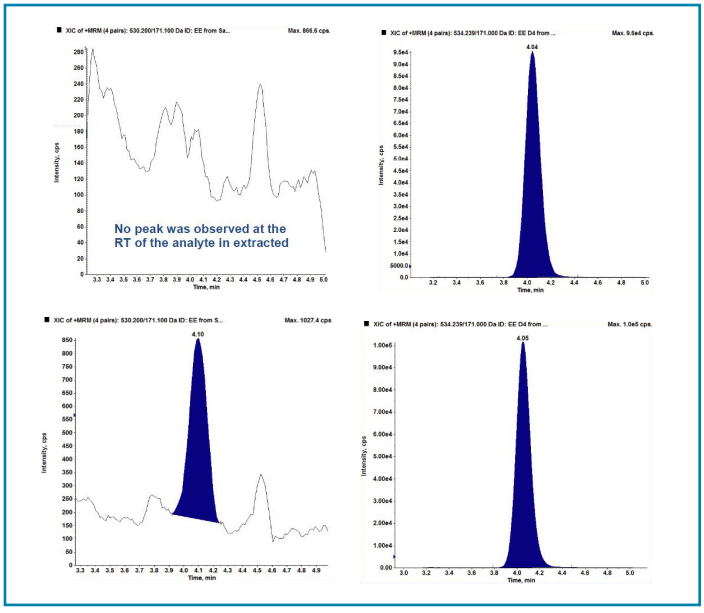

Figure 6: The chromatograms for Dansyl-Ethinyl Estradiol in zero blank plasma sample (upper pane) and 0.993 pg/mL spiked in plasma sample (lower pane)

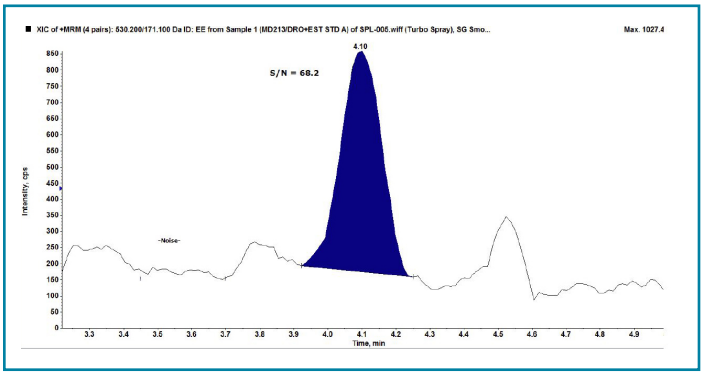

Figure 7: Signal to noise calculation for Dansyl-Ethinyl Estradiol in extracted plasma sample at LLOQ level (0.993 pg/mL) is 68.3

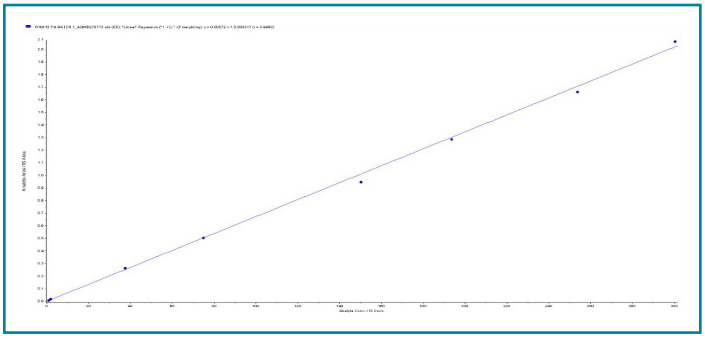

Figure 8: Calibration curve of Dansyl-Ethinyl Estradiol in plasma from 0.993 pg/ml to 300.485 pg/ml . The method has shown excellent linearity over the concentration range with r = 0.9980

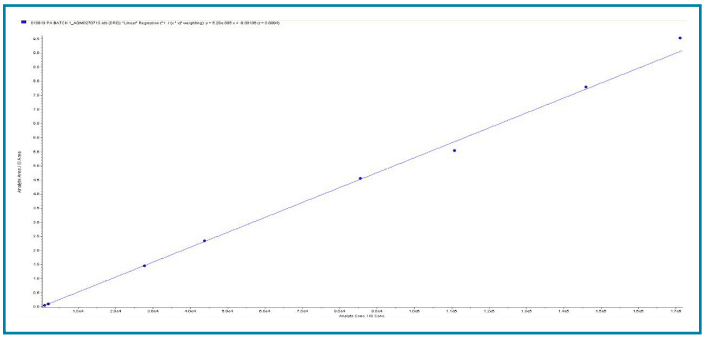

Figure 9: Calibration curve of drospirenone in plasma from 1003.63 pg/ml to 171152.24 pg/ml . The method has shown excellent linearity over the concentration range with r = 0.9994

Table 3: Full precision and accuracy batch data for Dansyl-Ethinyl Estradiol in plasma sample

Sample Name	Sample Type	Analyte Area	IS area	Area Ratio	Actual Con. (pg/ml)	Cal Con. (pg/ml)	Accuracy (%)
BLANK	Blank	0	0	0	0	0	0
BLANK DUP	Blank	0	0	0	0	0	0
ZERO BLANK	Double Blank	0	938116	0	0	0	0
ZERO BLANK DUP	Double Blank	0	947065	0	0	0	0
STD A	Standard	6774	1042864	0.0065	0.993	0.934	94.04
STD B	Standard	15652	1032269	0.0152	1.987	2.223	111.86
STD C	Standard	285956	1095472	0.261	37.486	38.791	103.48
STD D	Standard	571083	1139339	0.5012	74.971	74.516	99.39
STD E	Standard	1042239	1103685	0.9443	150.243	140.416	93.46
STD F	Standard	1416537	1102616	1.2847	193.646	191.04	98.65
STD G	Standard	1695288	1022132	1.6586	253.743	246.645	97.2
STD H	Standard	2294041	1114136	2.059	300.485	306.204	101.9
LLOQ QC -1	QC	7910	1067614	0.0074	0.998	1.07	107.18
LQC -1	QC	18848	1013308	0.0186	2.674	2.734	102.25
MQC -1	QC	1138887	1149211	0.991	150.243	147.36	98.08
HQC -1	QC	1810539	1135065	1.5951	246.398	237.204	96.27
BLANK-QC-1	Blank	0	0	0	0	0	0
LLOQ QC -2	QC	8023	1091544	0.0074	0.998	1.061	106.3
LQC -2	QC	20786	1107128	0.0188	2.674	2.76	103.22
MQC -2	QC	1145245	1153842	0.9925	150.243	147.588	98.23
HQC -2	QC	1842821	1155252	1.5952	246.398	237.214	96.27
BLANK-QC-2	Blank	0	0	0	0	0	0
LLOQ QC -3	QC	7561	1058303	0.0071	0.998	1.03	103.23
LQC -3	QC	21328	1132382	0.0188	2.674	2.769	103.55
MQC -3	QC	1138909	1146045	0.9938	150.243	147.77	98.35
HQC -3	QC	1776442	1117713	1.5894	246.398	236.35	95.92
BLANK-QC-3	Blank	0	0	0	0	0	0
LLOQ QC -4	QC	7327	1048775	0.007	0.998	1.007	100.88
LQC -4	QC	20886	1105252	0.0189	2.674	2.778	103.9
MQC -4	QC	1126520	1102080	1.0222	150.243	151.994	101.17
HQC -4	QC	1852672	1124222	1.648	246.398	245.066	99.46
BLANK-QC-4	Blank	0	0	0	0	0	0
LLOQ QC -5	QC	7443	1067437	0.007	0.998	1.005	100.68
LQC -5	QC	21344	1122133	0.019	2.674	2.797	104.59
MQC -5	QC	1181971	1162917	1.0164	150.243	151.133	100.59
HQC -5	QC	1892347	1152492	1.642	246.398	244.174	99.1
BLANK-QC-5	Blank	0	0	0	0	0	0
LLOQ QC -6	QC	7392	1060293	0.007	0.998	1.005	100.66
LQC -6	QC	22130	1148796	0.0193	2.674	2.833	105.94
MQC -6	QC	1174469	1158475	1.0138	150.243	150.749	100.34
HQC -6	QC	1859869	1109429	1.6764	246.398	249.299	101.18
BLANK-QC-6	Blank	0	0	0	0	0	0
LLOQ QC -1	QC	8202	1103094	0.0074	0.998	1.074	107.57
LQC -1	QC	18658	1004330	0.0186	2.674	2.731	102.12
MQC -1	QC	1152367	1150717	1.0014	150.243	148.909	99.11
HQC -1	QC	1814875	1124453	1.614	246.398	240.016	97.41
BLANK-QC-1	Blank	0	0	0	0	0	0
LLOQ QC -2	QC	7819	1156027	0.0068	0.998	0.974	97.57
LQC -2	QC	20760	1104472	0.0188	2.674	2.763	103.34
MQC -2	QC	1174976	1167045	1.0068	150.243	149.707	99.64
HQC -2	QC	1804859	1123271	1.6068	246.398	238.943	96.97
BLANK-QC-2	Blank	0	0	0	0	0	0
LLOQ QC -3	QC	8451	1057894	0.008	0.998	1.156	115.81
LQC -3	QC	22429	1138019	0.0197	2.674	2.899	108.41
MQC -3	QC	1140860	1130733	1.009	150.243	150.028	99.86
HQC -3	QC	1796568	1126934	1.5942	246.398	237.072	96.21
BLANK-QC-3	Blank	0	0	0	0	0	0
LLOQ QC -4	QC	7689	1046702	0.0073	0.998	1.06	106.24
LQC -4	QC	20964	1121431	0.0187	2.674	2.748	102.77
MQC -4	QC	1111755	1118328	0.9941	150.243	147.822	98.39
HQC -4	QC	1842642	1094014	1.6843	246.398	250.47	101.65
BLANK-QC-4	Blank	0	0	0	0	0	0
LLOQ QC -5	QC	7514	1059129	0.0071	0.998	1.023	102.49
LQC -5	QC	21109	1119136	0.0189	2.674	2.773	103.7
MQC -5	QC	1136565	1136572	1.0000	150.243	148.695	98.97
HQC -5	QC	1853768	1145757	1.6179	246.398	240.601	97.65
BLANK-QC-5	Blank	0	0	0	0	0	0
LLOQ QC -6	QC	7335	1032385	0.0071	0.998	1.024	102.64
LQC -6	QC	20904	1113784	0.0188	2.674	2.759	103.18
MQC -6	QC	1140076	1135968	1.0036	150.243	149.234	99.33
HQC -6	QC	1815216	1078222	1.6835	246.398	250.356	101.61
BLANK-QC-6	Blank	0	0	0	0	0	0

Table 4: Full precision and accuracy batch data for Drospirenone in plasma sample

Sample Name	Sample Type	Analyte Area	IS area	Area Ratio	Actual Con. (pg/ml)	Cal Con. (pg/ml)	Accuracy (%)
BLANK	Blank	0	0	0	0	0	0
BLANK DUP	Blank	0	0	0	0	0	0
ZERO BLANK	Double Blank	0	723630	0	0	0	0
ZERO BLANK DUP	Double Blank	0	744557	0	0	0	0
STD A	Standard	43512	827716	0.0526	1003.632	1019.248	101.56
STD B	Standard	86613	852938	0.1015	2007.263	1944.923	96.89
STD C	Standard	1269594	871988	1.456	27686.393	27544.06	99.49
STD D	Standard	2163306	924851	2.3391	43794.84	44235.05	101.01
STD E	Standard	4137593	909475	4.5494	85576.123	86011.23	100.51
STD F	Standard	5050928	912364	5.5361	110745.571	104659.3	94.5
STD G	Standard	6298565	808586	7.7896	145982.798	147251.4	100.87
STD H	Standard	8471100	889543	9.523	171152.246	180012.7	105.18
LLOQ QC -1	QC	46634	820920	0.0568	1004.007	1099.332	109.49
LQC -1	QC	114721	765308	0.1499	2706.219	2858.855	105.64
MQC -1	QC	3750300	837571	4.4776	84569.345	84653.37	100.1
HQC -1	QC	6465514	878482	7.3599	140948.909	139129.4	98.71
BLANK-QC-1	Blank	0	0	0	0	0	0
LLOQ QC -2	QC	46055	850857	0.0541	1004.007	1048.701	104.45
LQC -2	QC	136812	897367	0.1525	2706.219	2907.201	107.43
MQC -2	QC	3883127	860608	4.5121	84569.345	85305.11	100.87
HQC -2	QC	6441550	874753	7.3638	140948.909	139204.5	98.76
BLANK-QC-2	Blank	0	0	0	0	0	0
LLOQ QC -3	QC	44989	820538	0.0548	1004.007	1061.958	105.77
LQC -3	QC	127136	844085	0.1506	2706.219	2872.442	106.14
MQC -3	QC	3731672	836690	4.46	84569.345	84321.71	99.71
HQC -3	QC	6061461	807118	7.51	140948.909	141967	100.72
BLANK-QC-3	Blank	0	0	0	0	0	0
LLOQ QC -4	QC	43388	800670	0.0542	1004.007	1049.878	104.57
LQC -4	QC	129349	865128	0.1495	2706.219	2851.54	105.37
MQC -4	QC	3738489	853970	4.3778	84569.345	82766.89	97.87
HQC -4	QC	6596685	853086	7.7327	140948.909	146176.4	103.71
BLANK-QC-4	Blank	0	0	0	0	0	0
LLOQ QC -5	QC	46608	864451	0.0539	1004.007	1044.697	104.05
LQC -5	QC	128794	845264	0.1524	2706.219	2905.542	107.37
MQC -5	QC	4033150	897485	4.4938	84569.345	84960.43	100.46
HQC -5	QC	6493276	879457	7.3833	140948.909	139571.7	99.02
BLANK-QC-5	Blank	0	0	0	0	0	0
LLOQ QC -6	QC	46368	847928	0.0547	1004.007	1059.213	105.5
LQC -6	QC	134761	892569	0.151	2706.219	2879.25	106.39
MQC -6	QC	4051400	894376	4.5299	84569.345	85641.31	101.27
HQC -6	QC	6698465	872771	7.6749	140948.909	145084.3	102.93
BLANK-QC-6	Blank	0	0	0	0	0	0
LLOQ QC -1	QC	44202	831790	0.0531	1004.007	1030.06	102.59
LQC -1	QC	113168	746900	0.1515	2706.219	2889.395	106.77
MQC -1	QC	3810063	849549	4.4848	84569.345	84789.83	100.26
HQC -1	QC	6387153	853936	7.4797	140948.909	141393.4	100.32
BLANK-QC-1	Blank	0	0	0	0	0	0
LLOQ QC -2	QC	46977	871076	0.0539	1004.007	1044.958	104.08
LQC -2	QC	133094	887061	0.15	2706.219	2861.451	105.74
MQC -2	QC	3864471	856809	4.5103	84569.345	85271.69	100.83
HQC -2	QC	6271133	862339	7.2722	140948.909	137473	97.53
BLANK-QC-2	Blank	0	0	0	0	0	0
LLOQ QC -3	QC	45425	816638	0.0556	1004.007	1076.98	107.27
LQC -3	QC	128284	845304	0.1518	2706.219	2893.994	106.94
MQC -3	QC	3713134	827464	4.4874	84569.345	84838.17	100.32
HQC -3	QC	6060603	823800	7.3569	140948.909	139072.9	98.67
BLANK-QC-3	Blank	0	0	0	0	0	0
LLOQ QC -4	QC	41655	794816	0.0524	1004.007	1016.203	101.21
LQC -4	QC	128933	843980	0.1528	2706.219	2913.026	107.64
MQC -4	QC	3731948	845906	4.4118	84569.345	83409.52	98.63
HQC -4	QC	6316049	814215	7.7572	140948.909	146639.4	104.04
BLANK-QC-4	Blank	0	0	0	0	0	0
LLOQ QC -5	QC	46875	859705	0.0545	1004.007	1056.212	105.2
LQC -5	QC	124722	828209	0.1506	2706.219	2871.923	106.12
MQC -5	QC	3863058	875682	4.4115	84569.345	83404	98.62
HQC -5	QC	6227108	851013	7.3173	140948.909	138324.4	98.14
BLANK-QC-5	Blank	0	0	0	0	0	0
LLOQ QC -6	QC	43791	818559	0.0535	1004.007	1036.786	103.26
LQC -6	QC	131589	857424	0.1535	2706.219	2926.313	108.13
MQC -6	QC	3841234	865575	4.4378	84569.345	83901.02	99.21
HQC -6	QC	6548602	844062	7.7584	140948.909	146662.4	104.05
BLANK-QC-6	Blank	0	0	0	0	0	0

Conclusions

A highly sensitive method for Ethinyl Estradiol and Drospirenone was developed and validated using SCIEX Triple Quad™ 5500 LC/MS/MS system in human plasma. The developed method is sensitive, reproducible and cost effective for bioanalytical laboratory with good precision and accuracy

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