

Application Note

A Sensitive LC-MS/MS (API 4000) Method for Simultaneous Determination of Ethinyl estradiol and Levonorgestrel at LLOQ 2.00/25.0 pg/mL in Human Plasma

Introduction

Ethinyl estradiol is a semisynthetic alkylated estradiol with a 17-alpha-ethinyl substitution. It has high estrogenic potency when administered orally and is often used as the estrogenic component in oral contraceptives.

Levonorgestrel is a synthetic progestational hormone with actions similar to those of progesterone and about twice as potent as its racemic or (+)-isomer (norgestrel). It is used for contraception, control of menstrual disorders, and treatment of endometriosis.

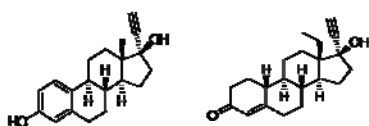


Figure 1. Structures of studied compounds: Ethinyl estradiol and Levonorgestrel resp.

The purpose of this work was to validate a sensitive high-performance HPLC method with the MS/MS detection for the simultaneous determination of ethinyl estradiol (2.00 – 300.00 pg/mL) along with levonorgestrel (25.0 – 10000.0 pg/mL) in human plasma. Following internal standards have been used: ethinyl estradiol-d₄ and levonorgestrel-d₇.

Experimental

Sample Preparation:

Blank human, fresh frozen K₂EDTA plasma was obtained from Sera Laboratories International Ltd UK. The material was stored at -20 °C ± 5 °C until the validation study was carried out.

Calibration standards were prepared at 8 none zero levels by fortifying blank plasma with ethinyl estradiol and levonorgestrel.

Sample Clean-Up:

The samples were carefully thawed at room temperature and prepared as follows:

- Thoroughly vortexed each sample prior to clean-up
- Took 500 µL of each plasma samples (calibration and QCs) into a 10-mL glass tube
- Added 25 µL of the mixed internal standard solution
- Liquid-liquid extraction with hexane/ethyl acetate in alkaline condition
- Shaked for 10 min
- Centrifuged 5 minutes with 4000 rpm at 5 °C
- Took organic layer to another 10-mL glass tube
- Evaporated organic layer (N₂, 40 °C)
- Derivatization with dansyl chloride and Na₂CO₃
- Vortexed and Incubated for 20 min at 50 °C in a drying oven
- Liquid-liquid extraction
- Shaked for 10 min
- Centrifuged 5 minutes with 4000 rpm at 5 °C
- Took organic layer to another 10-mL glass tube
- Evaporated organic layer (N₂, 40 °C)
- Reconstituted in 100 µL reconstitution solution
- Transferred reconstitution solution into a vial
- Crimp capped the vial
- Injected 20 µL for the LC-MS/MS analysis

Experimental

HPLC and Mass Spectrometric Parameters

HPLC-System Agilent 1100, CTC PAL Autosampler, coupled to a MS/MS-Spectrometer API 4000

Eluent:	0.1 % formic acid in water / acetonitrile (40/60; v/v)
Flow rate:	550 µL/min
Column temperature:	40 °C
Injection volume:	20 µL
Run time:	9.0 min

Ion transitions of the compounds and internal standards in the positive MRM-Mode:

ethinyl estradiol (dansyl chloride derivatized):	309.170 → 292.000
ethinyl estradiol-d ₄ (dansyl chloride derivatized):	314.297 → 297.900
levonorgestrel:	402.173 → 360.100
levonorgestrel-d ₇ :	405.254 → 361.000

Results and Discussion

Calibration

Table 1. Recalculated calibration standards with statistics of ethinyl estradiol (n=3)

C _{nominal} [pg/mL]	2.00	4.00	10.00	20.00	100.00	200.00	240.00	300.00
Mean	2.02	3.94	10.30	19.82	98.00	199.03	239.13	303.77
C _{measured} [pg/mL]	0.10	0.10	0.15	0.54	0.76	2.53	1.97	3.34
sd	4.8	2.5	1.5	2.7	0.8	1.3	0.8	1.1
cv [%]	1.0	-1.5	3.0	-0.9	-2.0	-0.5	-0.4	1.3
bias [%]	0.99977							
Mean r ²	0.99977							

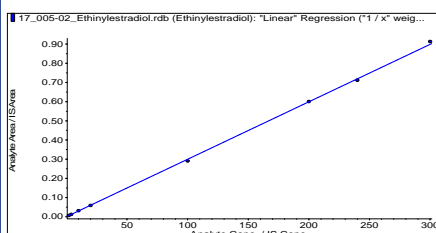


Figure 2. Calibration curve of ethinyl estradiol: Y = 0.003x + 1.04e-005 (r = 0.9999)

Table 2. Recalculated calibration standards with statistics of levonorgestrel (n=3)

C _{nominal} [pg/mL]	25.0	50.0	125.0	500.0	2500.0	5000.0	8000.0	10000.0
Mean	0.30	0.59	0.99	4.91	10.30	25.34	40.01	49.39
C _{measured} [pg/mL]	0.02	0.02	0.05	0.21	0.33	0.97	0.89	1.90
sd	6.9	3.4	5.5	4.3	3.2	3.8	2.2	3.8
cv [%]	0.2	-0.9	-1.3	-1.8	3.7	1.4	0.0	-1.2
bias [%]	0.99943							
Mean r ²	0.99943							

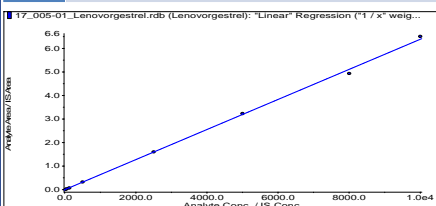


Figure 3. Calibration curve of levonorgestrel: Y = 0.000639X - 0.00285 (r = 0.9997)
Linearity of the method has been approved due to the correlation coefficients resulting in > 0.9900.

Matrix Effects

Table 3. Matrix Effects (n=12)

Analyte	ethinyl estradiol		levonorgestrel	
	IS normalized	IS normalized	IS normalized	IS normalized
C [pg/mL]	6.00	225.00	75.0	7500.0
mean [pg/mL]	1.03	0.96	0.93	0.99
sd	0.08	0.05	0.11	0.03
cv [%]	7.8	5.3	12.0	3.4

Matrix effects were investigated, using six lots of blank plasma from individual donors, one lot hemolytic and one lot lipedemic plasma. The IS-normalized matrix factor was calculated by dividing the MF of the analyte by the MF of the IS. The CV of the IS-normalized MF at LQC and HQC level is < 15 % and therefore in good accordance with the acceptance criteria.

Intra assay Accuracy and Precision

Table 4. Intra assay Accuracy and Precision (n=18)

Analyte	ethinyl estradiol			levonorgestrel				
	C _{nominal} [pg/mL]	6.00	90.00	225.00	25.0	75.0	3000.0	7500.0
Mean	1.99	5.84	87.56	218.59	27.3	75.8	3054.5	7875.3
C _{measured} [pg/mL]	0.19	0.38	1.65	4.59	3.5	4.7	93.7	219.2
sd	9.7	6.5	1.9	2.1	12.6	6.2	3.1	2.8
cv [%]	-0.5	-2.7	-2.7	-2.9	9.4	1.0	1.8	5.0
bias [%]	0.99977							

The intra assay precision and accuracy were calculated for each of the three validation sequences using six measured values at four concentrations.

Limit of Quantification

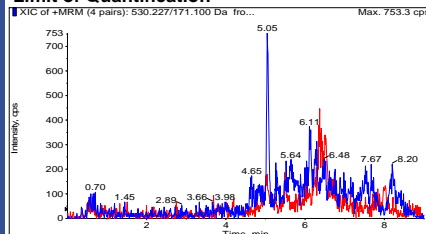


Figure 4. Chromatogram of ethinyl estradiol of the lowest calibrator (blue line) and a blank sample (red line), overlaid (RT = 5.05 min)

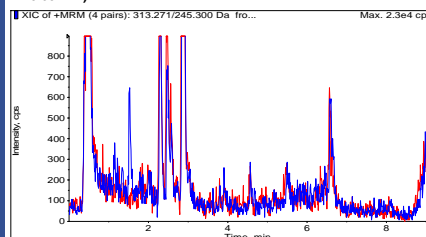


Figure 5. Chromatogram of levonorgestrel of the lowest calibrator (blue line) and a blank sample (red line), overlaid (RT = 1.52 min)

The calculated signal to noise ratio in the above chromatograms is > 5:1.

Conclusions

We successfully validated a very sensitive LC-MS/MS method for ethinyl estradiol and levonorgestrel at LLOQ 2.00/25.0 pg/mL in plasma with reliable accuracy and reproducibility and applied to BE study.

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