Quantification of Progesterone and 17-β Estradiol in Mouse Serum by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Introduction. *Chlamydia trachomatis* is a common sexually transmitted infection that can lead to severe secondary complications in women. A female's levels of estrogen and progesterone may play a role in the risk of chlamydial infection. This study aims to develop and evaluate an LC-MS/MS method for quantification of estrogen and progesterone in a mouse model for chlamydial infection (*Chlamydia muridarum)*.

Research Question. How accurate and reproducible is our LC-MS/MS method for quantifying estrogen and progesterone?

Study Design. The LC-MS/MS method was investigated for precision and accuracy of estradiol and progesterone quantification over three days. Several assay aspects were optimized for maximum analyte recovery and analytical sensitivity.

Methods. Progesterone samples were prepared using solid-liquid extraction (SLE+) and estradiol samples using liquid-liquid extraction (LLE) with subsequent derivatization. Hormones were quantified using LC-MS/MS with a gradient elution (C18 column) and direct ion channels for molecular ions for progesterone (m/z 315.01) and derivatized estradiol (m/z 431.24). Quantification was facilitated by deuterium-labeled internal standards and their corresponding molecular ions (d_9-progesterone; m/z 324.12 and d_5-estradiol; m/z 436.29).

Results. The dynamic range of the progesterone assay was 5–100 ng/mL, with a limit of detection of 1 ng/mL. The estradiol assay linear range was 5–100 ng/mL, with a limit of detection of 0.5 ng/mL. The average precision was 0.74–8.5%RSD and 6.3–13.4%RSD for progesterone and estradiol, respectively. The accuracy of the method was 1.6–14.4%Error and 4.0–10.5%Error for progesterone and estradiol, respectively. Successful validation was defined as <15% RSD and error (<20% at the limit of quantification), per current FDA Guidelines.

Conclusions. The developed LC-MS/MS method is specific for progesterone and estradiol, and the extraction suitable for preparation of mouse serum samples. This assay could be successfully applied to hormone quantification for investigating the link between chlamydia infection and hormone levels in female animals.

Methods

LC-MS/MS Parameters:

Column: UCT C18 (100 x 21 mm, 1.8 mm particle size) maintained at 50ºC

HPLC Gradient Elution: 1 mM ammonium fluoride in water and acetonitrile; 30%B ramp to 100%B over 10 minutes, hold at 100%B for 1 minute, re-equilibrate for 4 minutes at 30%B

Mass Spectrometry: Positive Electrospray

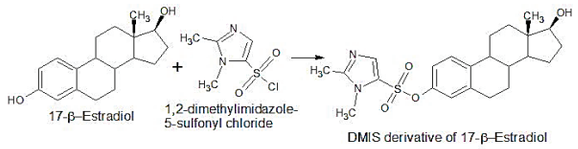
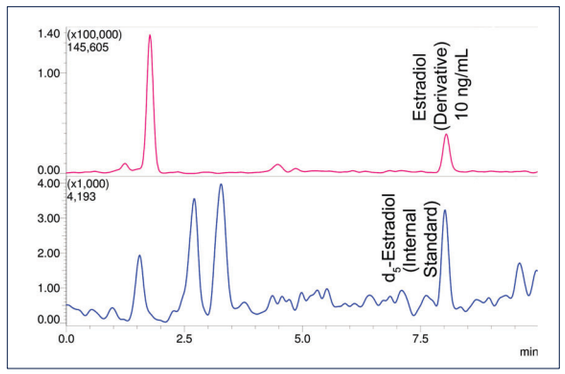


Figure 1: 17-β Estradiol Derivatization Reaction

Estradiol



Progesterone

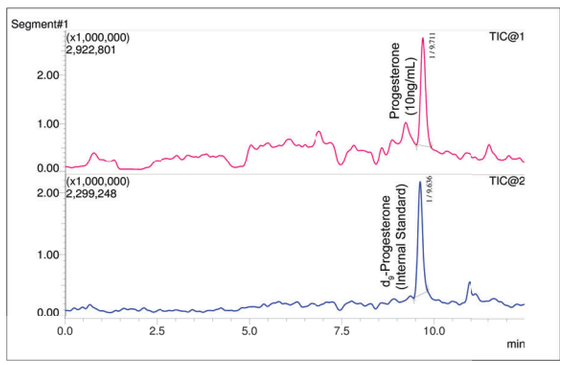


Figure 2: Mass chromatogram of derivatized estradiol (431.24 m/z), progesterone (315.01 m/z), and d_5-estradiol (436.29 m/z), and d_9-progesterone (324.12 m/z)

Estradiol Derivatization:

Sample Preparation
- One Eppendorf Tube
  - 10 µL of estradiol standard solution +
  - 10 µL of internal standard (conc. 100 ng/mL) +
  - 90 µL of MES buffer or plasma
  - Vortex

Liquid-Liquid Extraction
- Add 1 mL of methyl-t-butyl to the Eppendorf tube
- Vortex
- Transfer top aqueous layer to glass test tube for evaporation with N_2 gas at 37 ºC

Reconstitution and Measurement
- Glass test tube
  - 60 µL of sodium bicarbonate (pH 10.5) +
  - 40 µL of DIMS (derivatizing agent; see Figure 1)
  - Vortex
- Heat reconstituted solution in parafilm covered glass test tubes at 60ºC for 10 minutes for derivatization to occur
- Transfer solution to Eppendorf filter tube with Pasteur pipette
- Centrifuged for 3 minutes at 8300 rpm
- Transfer samples to glass vial with a Pasteur pipette for LC-MS analysis

Progesterone Extraction:

Sample Preparation
- One Eppendorf Tube
  - 10 µL progesterone standard solution +
  - 10 µL internal standard (conc. 100 ng/mL) +
  - 90 µL phosphate buffered saline (PBS) or plasma
  - Vortex
  - Add 100 µL LC-MS H_2O and vortex again

Solid-Liquid Extraction
- Load samples onto SLE+ cartridge followed by one pulse vacuum to load
- Wait 5 minutes
- Add 800 µL of ethyl acetate and gravity feed for 5 minutes
- Add 800 µL more of ethyl acetate and gravity feed for 5 minutes
- Follow with several vacuum pumps for full elution, do not let pressure exceed 10 mmHg
- Evaporate with N_2 gas at 37 ºC

Reconstitution and Measurement
- Add 80 µL acetyl nitrate
- Vortex
- Load samples into appropriate Eppendorf filter tube
- Centrifuge for 3 minutes at 8300 rpm
- Transfer to glass vials for LC-MS analysis

Figure 3: Estradiol derivatization and progesterone sample extraction to prepare samples for LC-MS analysis; for "real samples", the hormone standard solution is excluded.

Results

Estradiol

Concentration (ng/mL) Spiked	Average Calculated Conc	Standard Deviation	%RSD	Average % Error
100	99.11	2.93	2.96	1.91
50	45.06	2.03	4.51	9.86
20	20.44	1.92	9.43	7.16
10	10.21	0.81	8.00	6.76
5	5.56	0.24	4.33	11.35

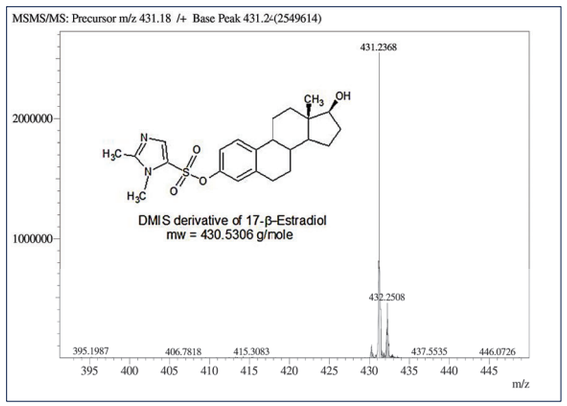
Progesterone

Concentration (ng/mL) Spiked	Average Calculated Conc	Standard Deviation	%RSD	Average % Error
100	102.83	6.45	6.27	4.02
50	52.37	4.74	9.06	7.47
20	20.79	1.09	5.28	4.20
10	10.39	0.89	8.57	6.10
5	4.71	0.63	13.44	10.53

Tables 1 & 2: Precision (%RSD) and accuracy (%Error) summaries for estradiol (top) and progesterone (bottom) quantification. Each concentration was repeated in triplicate.

Limit of Quantification (LOQ)** is highlighted in red for both charts. **Limit of Detection (LOD)** is 0.5ng/mL and 1ng/mL for estradiol and progesterone, respectively.

Estradiol



Progesterone

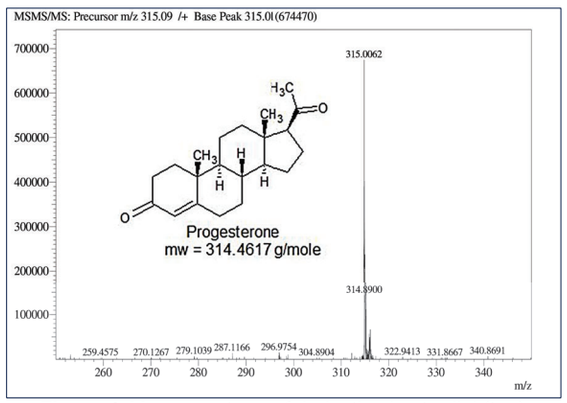


Figure 4: Mass spectrum of derivatized estradiol (top) and progesterone (bottom) showing m/z of molecular ion ($M+H^+$) used for quantification.

Conclusions

The developed LC-MS/MS method for quantification of estradiol and progesterone is precise and provides the ability to detect trace amounts of either hormone. This method will be utilized for hormone quantification of mouse serum samples in the future studies.

Acknowledgements

The authors would like to acknowledge the Bill Gatton College of Pharmacy and the Quillen College of Medicine for their ongoing support of student research.

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