

Reversed-Phase Liquid Chromatographic Method for Estrogen Determination in Equine Biological Samples

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Abstract

Equine unsaturated estrogens are the main components of brand formulations indicated for hormonal replacement therapy in both hypogonadic and postmenopausal women. These hormones are produced by the fetoplacental unit during equine gestation. A method is described for the quantitative determination of equilenin (EL), equilin (EQ), 17 α -dihydroequilin (17dEQ), and estrone (E1) in the plasma of a pregnant mare. Blood samples are obtained weekly during pregnancy by jugular venipuncture using sodium ethylenediaminetetracetic as the anticoagulant. For the quantitation of these estrogens, plasma is submitted to enzymatic hydrolysis followed by liquid-liquid extraction. A high-performance liquid chromatographic system equipped with a UV detector set at 220 nm and an ODS Hypersil column is used. The method met precision, specificity, and accuracy requirements. The hormonal levels determined in one target mare throughout pregnancy were 97.91 to 449.13, 116.47 to 266.02, 74.92 to 235.54, and 84.26 to 300.03 ng/mL, reaching a maximum towards the 25th, 20th, 33rd, and 27th weeks, respectively, for E1, EL, EQ, and 17dEQ. The method was successfully tested by quantitating these estrogens in the plasma from a pregnant mare. Its applicability to the study of estrogen bioavailability and bioequivalence is suggested.

Introduction

The pregnancy of mares (lasting on average 330 days) is characterized by a sequence of specific endocrine events differing from those observed in other domestic species and represents a model of the complexity of biological mechanisms. One of the peculiarities of equine pregnancy is the fact that the function of the fetoplacental unit is controlled by the fetal gonads and not by the

adrenals (as is the case for most species). These peculiarities have led to the intensive study of the endocrinology of a mare's pregnancy in an attempt to explain for what reason pregnancy is maintained by high estrogen levels. Estrogens are standard saturated compounds such as estradiol and estrone (E1) whose precursor is cholesterol in classic steroidogenesis. Also considered as estrogens are the unsaturated compounds equilin (EQ) and equilenin (EL) and the biologically active metabolites of EQ, among them 17 α -dihydroxyequilin (17dEQ), originating from alternative steroidogenesis and having farnesyl pyrophosphate as the primary precursor.

These estrogens have been frequently determined in biological samples by radioimmunoassay (RIA) (1–3). Some protocols propose the use of high-performance liquid chromatography (HPLC) as an auxiliary method for the separation of these substances (4–6), followed by quantitation by RIA. Some authors have used HPLC for the identification of the estrogens in rat, rabbit, and monkey plasma (7).

This study proposes a method for the quantitation of total (unconjugated and conjugated) EQ, EL, E1, and 17dEQ in pregnant mare plasma using reverse-phase HPLC with an ultraviolet detector (HPLC–UV) after enzymatic hydrolysis followed by liquid-liquid extraction.

Experimental

Chemicals and materials

EQ, E1, and 17dEQ were obtained from USP (Rockville, MD) and EL from Steraloids, Inc. (Wilton, NH). Methanol and acetonitrile were HPLC grade (EM Science, Gibbstown, NJ). Ether (Sinth, São Paulo, Brazil), sodium acetate (Sigma Chemical Co., St. Louis, MO), and acetic acid (Merck, Rio de Janeiro, Brazil)

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were analytical-reagent grade. The water used was freshly distilled, deionized, and purified with Milli Q plus equipment (Millipore, Bedford, MA). *Helix pomatia* S9751 was obtained from Sigma. The acetate buffer was pH 4.6 (43-mL 0.2M sodium acetate added to 57 mL of 0.2M acetic acid).

Preparation of standards

The standard solutions of EQ, E1, 17dEQ, and EL (1.0 and 0.1 mg/mL) were prepared in methanol.

Sample preparation

Extraction was performed by the method of Chandrasekaran et al. (7) and enzymatic hydrolysis by the method of Pashen et al. (8), both with slight modifications. Aliquots (1 mL) of plasma samples were acidified with 1 mL of acetate buffer (pH 4.6), and 500 IU of sulfatase (*Helix pomatia*) were added. After 1 h of incubation at 37°C, extraction was performed with 5 mL of ether with vigorous shaking for 20 s, and the preparation was centrifuged at 2000 rpm for 10 min. The organic layer was evaporated to dryness under a nitrogen flow. The residues were reconstituted in 200 μ L of mobile phase, filtered through a reduced cellulose syringe filter (0.45- μ m pore size, 13-mm i.d.) (Hewlett Packard, Weiterstadt, Germany), and 50 μ L was injected into the column.

Apparatus and chromatographic conditions (HPLC–UV)

A Hewlett Packard (Palo Alto, CA) 1100 liquid chromatograph equipped with a UV detector set at 220 nm was used. An ODS-Hypersil column (125 \times 4 mm, 5- μ m particle size) (Hewlett Packard) was kept below 40°C throughout the analysis. The water–acetonitrile mobile phase (60:40, v/v) was isocratic and

used at a flow rate of 1.0 mL/min for 6.8 min. At 7.5 min the mobile phase was changed to 100% acetonitrile. This composition was maintained for 1 min at a flow rate of 1.5 mL/min. At 9 min the starting mixture was used again and maintained up to 11 min. The injection volume was 50 μ L.

Calibration curve

The calibration curves for the quantitation of equine estrogens were constructed with negative control samples spiked with six different concentrations of 17dEQ, EQ, EL, and E1, according to the linearity range following the proposed method without hydrolysis. The curves were obtained using the peak area from each estrogen versus its different concentrations.

Linearity and sensitivity

Linearity was studied by the repeated analysis ($n = 3$ –6) of EQ, E1, 17dEQ, and EL using different concentrations of these estrogens in the 40–1000-ng/mL range. The coefficient of variation (CV) was determined for each concentration, and the limit of quantitation was determined as the lowest concentration at which the CV was 15% or less.

Precision and accuracy

Intra-assay data were obtained using negative control samples spiked with different concentrations (70–1000 ng) by replicate analysis ($n = 2$) evaluated for the linearity of EQ, E1, 17dEQ, and EL. Interassay variability was assessed on the basis of six day determinations. The accuracy of the method was defined as the percent difference between the mean concentration found and the theoretically expected concentration ($n = 3$).

Negative control sample

Blood samples were obtained from an 18-year-old gelding horse by venipuncture using sodium ethylenediaminetetracetic (EDTA). Plasma was identified, stored at -20°C , and used as a biological matrix for method validation and the calibration curve. The samples were submitted for the same HPLC–UV method after a sample preparation step showing negative results for steroids.

Applicability

The applicability of the method was tested by analyzing samples from a target pregnant mare as an experimental model. Blood was obtained weekly during gestation by jugular venipuncture using EDTA, and plasma was identified and stored at -20°C until the time for assay.

Table I. Work Range, Equation of the Line*, and Correlation Coefficient for the Method Developed for the Quantitation of 17dEQ, EL, EQ, and E1 in Equine Plasma

	17dEQ	EL	EQ	E1
Work range (ng/mL)	40–750	50–750	50–800	40–1000
a	0.4334	2.0768	0.2965	0.2601
b	-11.712	-28.852	-5.882	-4.929
r [†]	0.9974	0.9965	0.9940	0.9983

* $y = ax + b$.

[†] r, correlation coefficient.

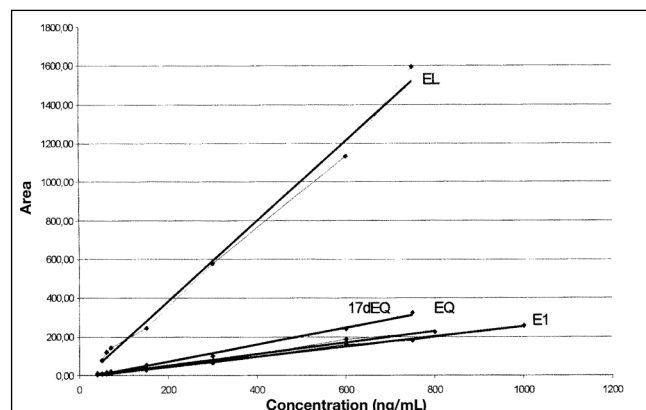


Figure 1. Graphic presentation of the linearity obtained with the proposed method for 17dEQ, EL, EQ, and E1.

Results and Discussion

In view of the unavailability of commercial RIA kits for equine estrogens (EQ, 17dEQ, and EL), the difficulty in producing specific antibodies, and the high percentage of cross-reactions with substances having a similar chemical structure (9–11), it has become necessary to develop a safe and accurate method that will permit the quantitation of these sex steroids.

Estrogens circulate in the bloodstream in the unconjugated and conjugated form (sulfate and glucuronate). However, most of the components in blood are in the sulfated form (12), justifying the option for enzymatic hydrolysis with a sulfatase.

Chromatographic separation proved to be selective, with retention times of 4.3, 4.9, 5.3, and 5.8 min for 17dEQ, EL, EQ, and E1, respectively. The method was linear for the concentration ranges of 40 to 750, 50 to 750, 50 to 800, and 40 to 1000 ng/mL for 17dEQ, EL, EQ, and E1, respectively (Figure 1), with a correlation coefficient of more than 0.994 (Table I). The specificity of the method was demonstrated by the absence of endogenous peaks coeluting with the estrogens under study (Figure 2).

The intra-assay CV for the analysis of two daily determinations evaluated for six different days was 2.27–16.46% within the concentration range studied, and the interassay precision observed on the same days (demonstrated by the CV) ranged from 0.87% to 17.73%, as shown in Table II. The percent variation assessed by evaluating the accuracy for a specific concentration (300 ng/mL)

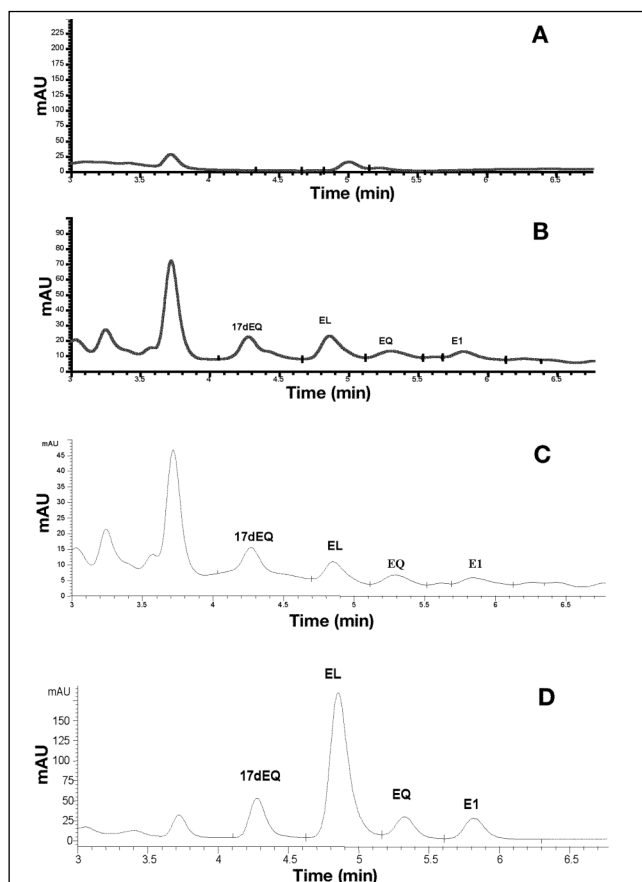


Figure 2. Representative chromatograms obtained during quantitative estrogen analysis by HPLC for a pregnant mare: (A) negative control, (B) 33rd week of gestation, (C) 40th week of gestation, and (D) calibration curve level.

Table II. Results Obtained for the Analytical Precision of the Determination of 17dEQ, EL, EQ, and E1 in Equine Plasma

Estrogen	Concentration range (ng/mL)	Intra-assay precision (%CV)	Interassay precision (%CV)
17dEQ	70–750	4.15–11.81	7.87–16.11
EL	70–750	2.37–9.18	2.50–11.41
EQ	70–800	4.20–16.46	3.16–9.68
E1	70–1000	2.27–9.17	0.87–17.73

was –0.06%, –9.18%, 14.84%, and 16.07% for 17dEQ, EL, EQ, and E1, respectively.

Figure 2 shows the chromatograms obtained by HPLC–UV analysis. Using this technique it was possible to determine the hormonal profile of pregnancy for the mare chosen as a “model” representative of the study. The estrogen concentrations ranged from 97.91 to 420.00, 117.73 to 179.02, 74.92 to 208.89, and 84.26 to 300.03 ng/mL, reaching maximum values during the 5th, 7th, 6th and 6th month for E1, EL, EQ, and 17dEQ, respectively.

Conclusion

The method proved to be rapid and accurate and has been successfully used in quantitating the availability of equine estrogens for the fetoplacental unit in pregnant mares. The method could be adapted to the study of estrogen bioavailability and bioequivalence.

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