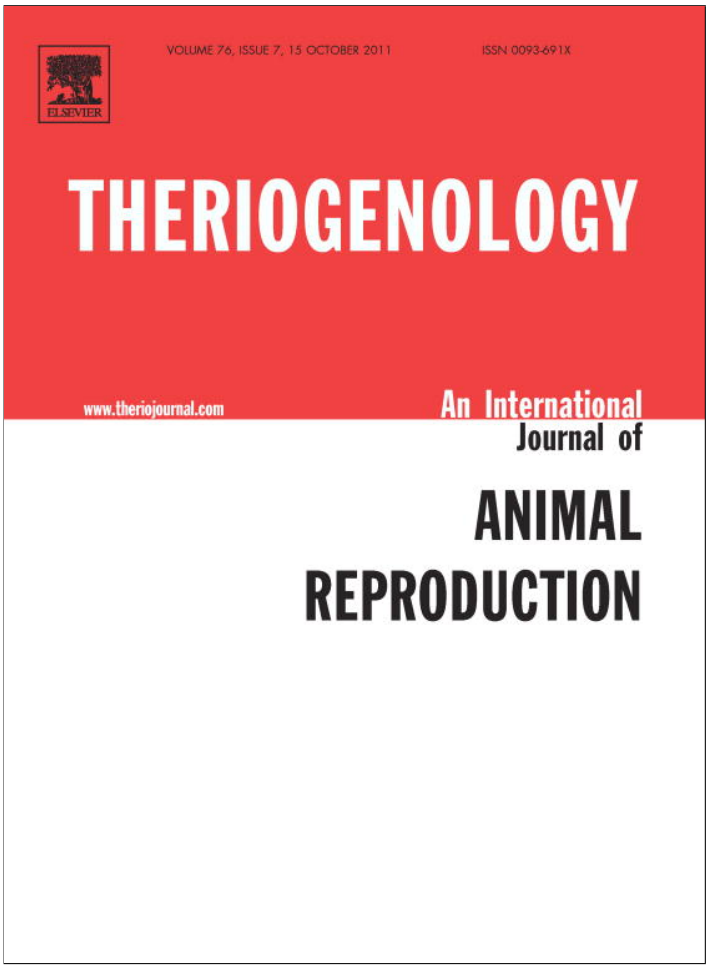

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LC-MS/MS quantitation of plasma progesterone in cattle

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Abstract

Quantitation of progesterone (P_4) in biological fluids is often performed by radioimmunoassay (RIA), whereas liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been used much less often. Due to its autoconfirmatory nature, LC-MS/MS greatly minimizes false positives and interference. Herein we report and compare with RIA an optimized LC-MS/MS method for rapid, efficient, and cost-effective quantitation of P_4 in plasma of cattle with no sample derivatization. The quantitation of plasma P_4 released from three nonbiodegradable, commercial, intravaginal P_4-releasing devices (IPRD) over 192 h in six ovariectomized cows was compared in a pairwise study as a test case. Both techniques showed similar P_4 kinetics ($P > 0.05$) whereas results of P_4 quantitation by RIA were consistently higher compared with LC-MS/MS ($P < 0.05$) due to interference and matrix effects. The LC-MS/MS method was validated according to the recommended analytical standards and displayed P_4 limits of detection (LOD) and quantitation (LOQ) of 0.08 and a 0.25 ng/mL, respectively. The high selective LC-MS/MS method proposed herein for P_4 quantitation eliminates the risks associated with radioactive handling; it also requires no sample derivatization, which is a common requirement for LC-MS/MS quantitation of steroid hormones. Its application to multisteroid assays is also viable, and it is envisaged that it may provide a gold standard technique for hormone quantitation in animal reproductive science studies.

Keywords: Bovine; Progesterone; LC-MS/MS; Radioimmunoassay; Estrus synchronization

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1. Introduction

Synchronization of estrus in cows is one approach to improve animal production efficiency, and progesterone (P_4) is a steroid hormone widely used for such a goal by means of intravaginal delivery systems, generally in combination with estrogens and luteolytic drugs. Quantitation of P_4 in biologic fluids is therefore fundamental for the efficacy of biotechnologies for synchronization of estrus in cattle and this important analytical task has largely been performed via radioimmunoassay (RIA). Despite the risks associated with handling radioactive materials, RIA is relatively simple, rapid, sensitive, and easy to perform [1]. Many studies have, however, indicated that RIA exhibits poor specificity for steroids with several interferences and severe matrix effects. Antibodies used for RIA can cross-react during the recognition of steroids, which are present in low concentrations in complex biological fluids [2-4]. Steroid hormones have been also been quantitated with chromatography [4] and gas chromatography-mass spectrometry (GC-MS) [5-7].

Mass spectrometry (MS) is currently considered the analytical technique of choice for the secure identification and quantitation of drugs and hormones in human clinical practice and residues in food [8,9]. For MS analysis**,** analyte molecules are ionized, and the resulting ions are separated according to their mass-to-charge (*m/z*) ratios, followed by detection and counting via ion current measurements. The ion current generates an electric pulse that forms a peak in the mass spectrum. Therefore, an MS spectrum displays *m/z* values as a function of abundances of the detected ions [10]. Ions of interest can also be dissociated to generate specific fragment ions via tandem MS/MS experiments for more secure identification (higher selectivity) and improved signal-to-noise ratios for quantitation [11].

By coupling liquid chromatography (LC) to tandem mass spectrometry (MS/MS), high analytical specificity is achieved due to the combined use of the three selective parameters: (1) chromatographic retention time; (2) monitoring the specific *m/z* of the precursor ion; and (3) additional monitoring of the *m/z* of a single of characteristic set of product ions [12]. For quantitation of steroid hormones, LC-MS/MS with atmospheric pressure photoionization (APPI) is considered the most efficient platform [11,12].

The objective of this work was to develop an efficient LC-MS/MS method that would require no derivatization procedures for determination of plasma P_4 concentrations in cattle. Atmospheric pressure photoionization (APPI) is an efficient ionization method for steroids [12]; hence APPI was selected for best performance. The LC-MS/MS method was validated according to the recommended analytical standards [8] and then compared with RIA results. Quantitation of plasma P_4 released by three silicone, intravaginal P_4-releasing devices (IPRD) was used as a test case.

2. Methods

2.1. Chemicals and solvents

Standards for P_4 and medroxyprogesterone 17-acetate (MPA) were purchased from Sigma Aldrich (St. Louis, MO, USA), whereas high pressure liquid chromatography (HPLC)-grade methanol, toluene, and hexane were purchased from Burdick & Jackson (Muskegon, MI, USA).

2.2. Experimental design and plasmatic samples

Six ovariectomized (OVX) *Bosindicus* cows 6 yr of age and 574.8 ± 10.7 kg were used. The cows had been OVX 2 yr prior to the start of the study. During the study, the cows grazed in a *Brachiariabrizanta* pasture and received water and mineral supplementation ad libitum. The study was approved by the Bioethical Committee of the FMVZ - University of São Paulo, Pirassununga, SP, Brazil, under protocol 1761/2009.

The cows received an IPRD for 7 days, to provide progesterone priming. Five days after removal of the IPRD, the animals were randomly assigned to receive one of three commercially available IPRD that contained 1g of P_4 (designated as devices A, B, and C). Device A was Sincrogest (OuroFinoSaude Animal; Cravinhos, SP, Brazil), device B was Cronipress (Biogenesis-Bago, Garin, Argentine), and device C was Primer (Grascon do Brasil Ltda; Sao Paulo, SP, Brazil). Each cow received only one IPRD, and each IPRD was designated to two animals. Blood samples were collected before device implantation (0 h) and at 2, 4, 6, 8, 10, 12, 24, 48, 72, 96, 120, 144, 168, and 192 h after implantation, using EDTA as an anticoagulant (Vacuette K3 EDTA, Greiner Bio-one; Americana, SP, Brazil). Samples were immediately centrifuged at 1500 × g at 4°C for 15 min in a RC3 Plus Centrifuge (Sorvall; Wilmington, DE, USA) and were stored at −20°C until use for P_4 quantitation by LC-MS/MS and RIA. The same samples were quantitated by both methods.

2.3. Standards and sample preparation

Stock (100 μg/mL) and working standard solutions were prepared in methanol.

For analytical calibration, plasma solutions contained P_4 in six concentrations (0.25, 1.0, 2.5, 5.0, 7.5, and 10 ng/mL) and the internal standard (2.5 ng/mL). Validation calibrators, quality control samples (QCs), and blanks were made using bull plasma that was filtered in activated charcoal, considered a biological matrix free from the analyte of interest [13].

The liquid phase extraction with hexane (1000 μL) was used for plasma samples (400 μL) that contained the internal standard solution. The samples were extracted for 30 sec using a vortex, and then centrifuged for 3 min at 14 000 × g (Eppendorf Minispin Centrifuge; Hamburg, Germany). The supernatant (700 μL, organic phase) was collected and evaporated to dryness under a N_2 stream. Samples were reconstituted in methanol (250 μL) prior to analysis in an LC-MS/MS system.

2.4. Chromatographic and mass spectrometer conditions

Chromatographic separations were performed using an 1100 series high pressure liquid chromatography (HPLC) system (Agilent Technologies, Minnetonka, MN, USA) with an Agilent Eclipse column (XDB-C18 5 μm, 4.6 × 150 mm) at 30°C. Methanol was used as the mobile phase at a flow rate of 0.8 mL/min. The total run time was 3.5 min.

Mass spectrometric analysis was performed using a 4000 QTrap tandem mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with an APPI source, which was operated in the positive ion mode. The source was also equipped with a 10 eV krypton discharge lamp and dopant (toluene) delivery set to 100 μL min^{-1}. Nitrogen was used as curtain (10 psi), nebulizer (40 psi), auxiliary (40 psi), and collision (9 arbitrary units - a.u.) gases. The ion transfer voltage was set to 800 V, and the probe temperature to 350°C. At these settings, P_4 and MPA were detected in their protonated form ($[M + H]^+$). Sample analysis was carried out in the multiple-reaction monitoring (MRM) mode with a dwell time of 75 ms per channel using the following *m/z* transitions: 315.20 > 109.20 for P_4 quantitation, 315.20 > 123.20 for P_4 confirmation, and 387.20 >123.10 for MPA quantitation. System control and data acquisition were performed using Analyst software (Version 1.5, AB Sciex).

2.5. P_4 quantitation by RIA

For RIA, plasma P_4 concentrations were determined using a commercial radioimmunoassay kit (Coat-A-Count, Siemens; Los Angeles, CA, USA) according to manufacturer's instructions. Standards and pooled aliquots of bovine plasma were done in parallel with plasma volumes ranging from 50 to 200 μL. Accuracy was tested by adding 100 μL from a previously assayed bovine plasma pool (4.6 ± 0.2 ng/mL) to the standards.

2.6. Statistical analysis for RIA and LC-MS/MS comparison

To assess statistical differences, a split-split-plot ANOVA using time as a subplot and method as a sub-subplot was performed. Variables considered in the model were IPRD (A, B, or C), animals (repetitions), time (0 to 192 h), methods (LC-MS/MS and RIA), and interactions. Means were compared by Student *t* test. A level of 5% significance was used. Analyses were performed using Statistical Analysis System version 8.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. LC-MS/MS method development and validation

In the MS data for P_4 standard solution (Fig. 1A), P_4 was detected as its protonated molecule of *m/z* 315. The APPI-MS/MS data for the $[P_4 + H]^+$ ion of *m/z* 315 shows that it dissociates to form two major fragment ions of *m/z* 109 and 123 (Fig. 1B). Williams et al. [14] have proposed mechanisms leading to these fragment ions, based on comparison with other similar steroid hormones.

3.1.1. Specificity

Specificity was examined by analyzing five pools of bull plasma previously filtered in activated charcoal to remove steroids. There were no significant interferences at the retention time of the drug or internal standard (Fig. 2).

3.1.2. Calibration curve and linearity

Calibration curves were constructed by linear fitting, using the least squares linear regression calculation with no weighting. The ratio of mean peak area of P_4 to MPA was linearly related to the concentration of P_4 (R^2 = 0.999) over the range of 0.25 to 10 ng/mL.

3.1.3. Limits of detection and quantitation

Using the proposed methodology, the limit of detection (LOD) determined for P_4 was 0.08 ng/mL, considering a signal-to-noise ratio ≥ 3. The limit of quantitation (LOQ) was defined as the lowest concentration in the calibration curve where the analyte could be detected with an acceptable accuracy (relative error ≤ 20%), precision (relative standard deviation, defined as the ratio of standard deviation to the mean, multiplied

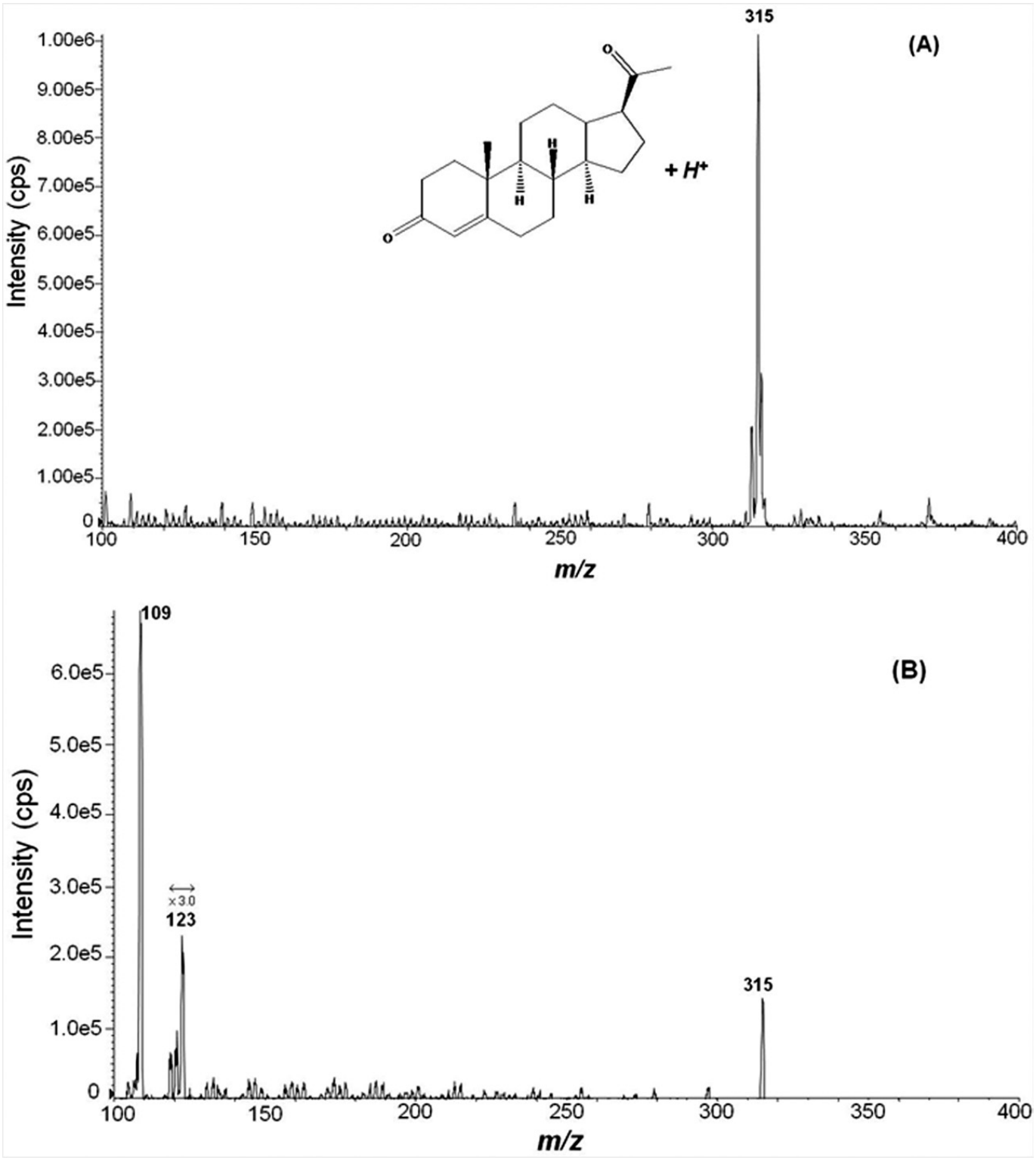

Fig. 1. (A) Representative atmospheric pressure photoionization (APPI) mass spectrum of a methanolic solution of progesterone (P_4). The ion of *m/z* 315 corresponds to $[P_4 + H]^+$, whereas the inset presents the molecular structure of P_4. (B) APPI-mass spectrometry [MS]/MS of $[P_4 + H]^+$ of *m/z* 315. Note the major fragments ions of *m/z* 109 and 123 which were used to secure quantitation of P_4 by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

by 100; relative standard deviation [RSD] ≤ 20%), and a signal-to-noise ratio ≥ 10. According to these conditions, the LOQ for P_4 was 0.25 ng/mL (RSD = 5.7%).

3.1.4.Accuracy and precision

Accuracy was defined by the measured concentration represented as a percentage of the expected con-

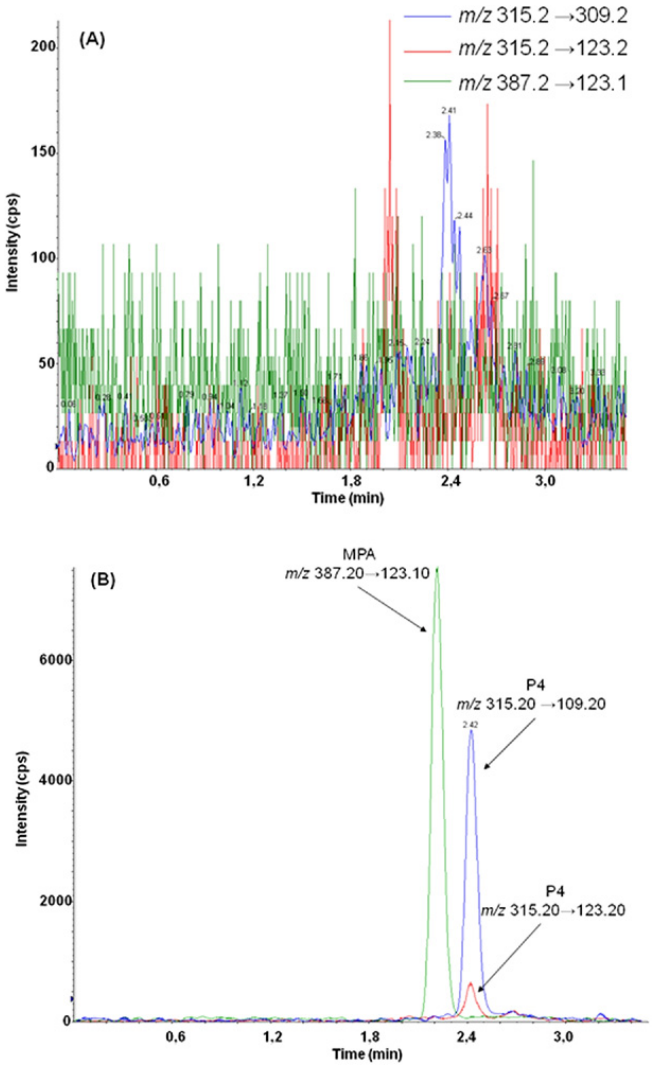

Fig. 2. Chromatograms showing the selectivity of the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method for progesterone (P_4) quantitation. (A) A blank plasma extraction and (B) a plasma extract that contains P_4 and medroxyprogesterone 17-acetate (MPA) at the limit of quantitation (LOQ) are represented.

centration. The precision and accuracy of the LC-MS/MS method was determined by analysis of low, medium, and high QC samples at three concentrations (0.5, 4.0, and 8.0 ng/mL). Injecting five samples of each concentration in the same day assessed the intraday variation of the assay, and interday assay variation was assessed by the injection of five samples of each concentration on Day 3. The limits for the accuracy of values were set as the range, 85% to 115%, except at the LOQ, where values between 80% and 120% were accepted. The % RSD of the regressed (measured) concentrations was used to report precision. Precision for all concentrations was accepted if the % RSD fell within ± 15%, except at the LOQ, where the limit was extended to ± 20%. Intra- and interassay accuracies at the LOQ and QC concentration are shown (Table 1). Precision and accuracy values considered in this work were according to the guidance for bioanalytical method validation [15].

3.1.5.Recovery and matrix effect

The analyte recovery and the matrix effect were investigated at three concentrations (2.5, 5.0, and 7.5 ng/mL). The average recoveries of P_4 obtained for the tested concentrations were 87%, 89%, and 83%, respectively.

The matrix effect was investigated by comparing the peak areas of the standard solutions prepared in methanol with blank plasma extracts spiked at the same nominal concentrations. As a result, the ion signal suppression was found to be negligible for the analyte detection, as the blank matrix-matched standards areas were slightly smaller than the methanol standards. The matrix effects were, 8.0%, 6.7%, and 1.3% for concentrations of 2.5, 5.0, and 7.5 ng/mL, respectively.

3.2. Quantitation of plasma P_4 by LC-MS/MS in OVX cows with polymeric devices

The P_4 profiles obtained for the three devices were similar ($P > 0.05$). Fig. 3 shows the kinetics of plasma P_4 concentrations measured by LC-MS/MS, following the intravaginal insertion of the three IPRD loaded with P_4 during fixed intervals over 192 h. The maximum concentration of P_4 was ob-

Table 1
The intra- and interassay precision and accuracy variation for P_4 quantitation with LC-MS/MS.

P_4 (ng/mL)	Intra-assay (N = 5)			Interassay (N = 15)		
	Mean ± SD	RSD (%)	Accuracy (%)	Mean ± SD	RSD (%)	Accuracy (%)
0.25	0.25 ± 0.02	5.7	98.2	0.25 ± 0.03	10.0	97.9
0.50	0.45 ± 0.01	2.3	90.0	0.46 ± 0.02	3.4	92.6
4.00	3.98 ± 0.2	4.4	99.5	4.0 ± 0.2	5.3	99.8
8.00	8.3 ± 0.2	2.4	103.2	8.2 ± 0.4	5.0	101.9

LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; P_4, progesterone; RSD, relative standard deviation.

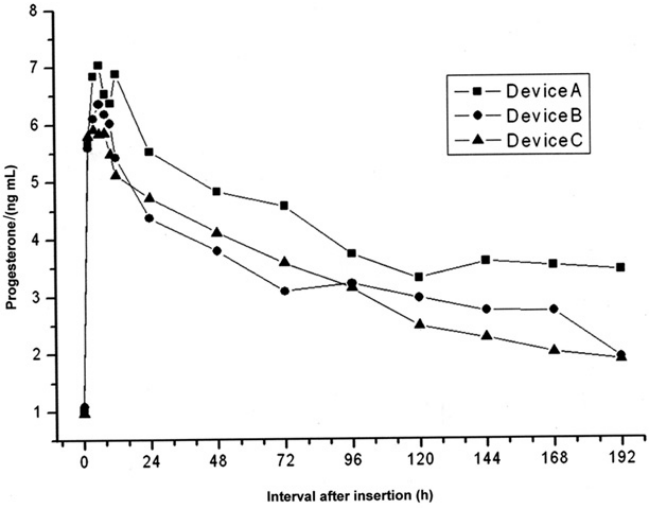

Fig. 3. Mean values of progesterone (P_4) concentration provided by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in three pairs of cows; each pair had an intravaginal progesterone-releasing device (A, B, and C) in place for 192 h ($P > 0.05$). Quantitation data are reported in Supplementary Tables 1–3.

served at 4-6 h (Table 2). All quantitation data are reported in Supplementary Tables 1–3.

3.3. Radioimmunoassay analytical parameters

For the RIA standard curve, measured values averaged 96.3 ± 1.1% of the predicted concentrations. The sensitivity of the assay, as defined as 93% of total binding, was 0.003 nmol/L. The intra- and interassay coefficients of variation were 2.03% and 4.72%, respectively.

3.4. Comparison of LC-MS/MS and RIA P_4 quantitation results

To compare RIA and LC-MS/MS results, aliquots from the same samples were submitted to both P_4 quantitation assays. As for LC-MS/MS, no significant differences among the three devices were detected using RIA. Comparison for the three IPRD of both methods (Fig. 4) confirmed that the P_4 kinetics was similar for all devices ($P < 0.05$).

Table 2
Data obtained by LC-MS/MS for maximum plasma P_4 concentrations (C_{max}) and times to reach these maxima (t_{max}) when released from three progesterone-releasing intravaginal devices.

Intravaginal device	C_{max} (ng/mL)	t_{max} (h)
A	7.04	6
B	6.36	6
C	5.91	4

LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; P_4, progesterone.

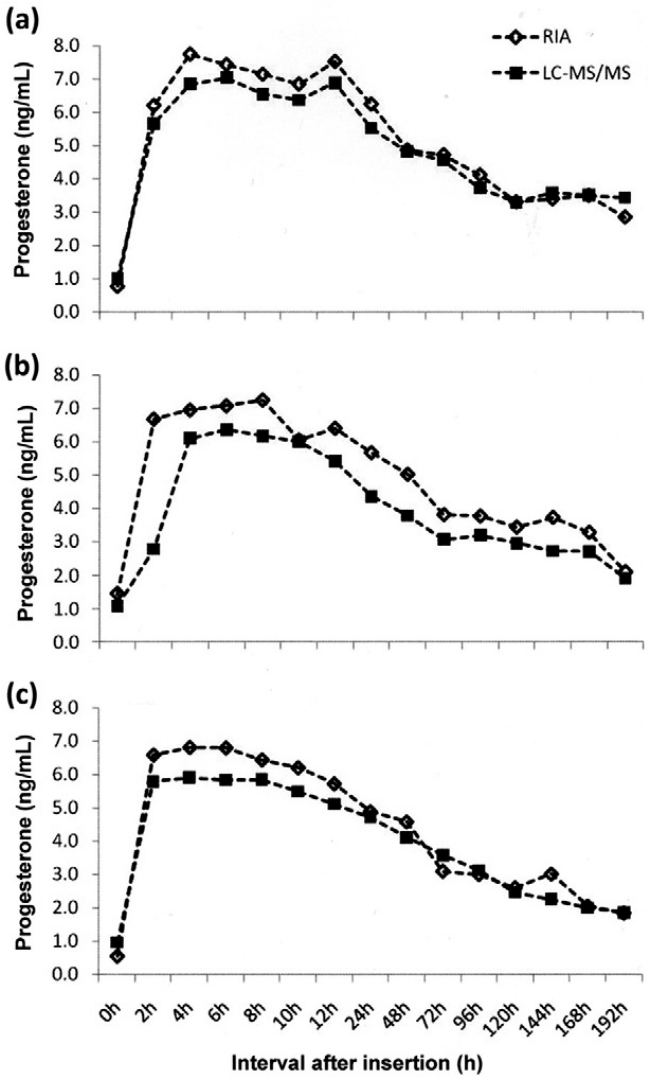

Fig. 4. Mean values from the comparison of radioimmunoassay (RIA) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) progesterone (P_4) quantitation in plasma samples from cows (N = 2) with an intravaginal progesterone releasing device A (a), B (b), or C (c) in place for 192 h. Quantitation data are reported in Supplementary Tables 1–3.

Progesterone concentrations quantitated by RIA (RSD 14.49%) were significantly higher than the concentrations obtained by LC-MS/MS (RSD 21.76%) and this bias was consistently present over all assayed times.

4. Discussion

This work describes the results from critical validation, showing high analytical confidence for the LC-MS/MS quantitation of P_4 in bovine plasma when APPI was used as the ionization technique. Using liquid extraction, sample preparation was simple and rapid, and P_4 quantitation by LC-MS/MS was also cost-effective.

This protocol may therefore be applicable for the high-throughput analyses of large sample sets, which is common in studies involving cattle. The LC-MS/MS method for evaluation of P_4 release from three commercial IPRD devices has also been compared with results from RIA analyses for the same samples.

Due to commercial and scientific reasons, there is increasing demand in human and veterinary endocrinology for accurate quantitation of steroid hormones in various biological matrices. Because steroid hormones occur in very low concentrations (e,g., ng/mL or pg/mL), these assays demand the use of analytical techniques of high sensitivity and selectivity [6,16,17].

Even though similar P_4 kinetics were detected by both techniques, RIA quantitation values were greater than those obtained by LC-MS/MS. This discrepancy may be attributed to interferences and matrix effects for RIA quantitation. Immunoassay techniques are commonly employed to quantitate steroid hormones in biological samples, due primarily to their simplicity, but their selectivity are limited by an intrinsic lack of steroid specificity due to cross-reactivity [4]. This poor steroid specificity seems to result from the common reaction of the phenanthrene ring structure of the antibodies with a pentane ring attached in the steroid hormone structure, as well as severe matrix effects resulting from inefficient extraction from complex biological matrices [17,18]. The lack of a gold standard analytical technique and its proper validation in steroid hormones assays is therefore considered a major flaw in epidemiological studies. Because immunoassay methods vary in efficiency, published results with high variability and inconsistent scientific interpretations remain, and immunoassay cross-reactivity has been reported to overestimate the steroid amount by as much as 60% or more [19-21].

The RSD of LC-MS/MS was slightly higher (21.76%) than that for RIA (14.49%), but this variation was attributed to the LC-MS/MS repetitions being performed by three researchers on three different days, whereas the RIA assay was performed on the same day by the same researcher.

Confidence of an analytical method is accessed via validation procedures that are demanded by regulatory agencies worldwide. Analyte recovery, limits of detection, LOQ, specificity, accuracy, and precision are the most common analytical parameters used for proper analytical validation [15,22]. The proposed LC-MS/MS method of P_4 quantitation has therefore been validated using all these test parameters. After testing different samples using various extraction protocols, it was found that liquid phase P_4 extraction was a rapid and inexpensive sample preparation protocol suitable for the large numbers of samples in P_4 quantitation studies with cattle. The use of this rapid and cost-effective sample extraction protocol, based on a simple liquid extraction, also facilitated greater analyte recovery rates (80% to 87%) and minimal matrix effect ($<$ 10%). The P_4 linear ranges were also satisfactory (0.25 to 10 ng/mL) with LOQ of 0.25 ng/mL. High accuracy (98.2% and 97.9% for intraday and interday variation, respectively) was also attained.

The LC-MS/MS methods for steroid hormone quantitation have required derivatization procedures to improve analyte ionization efficiency [22,23]. In bovine plasma, P_4 has been quantitated by LC-MS/MS with electrospray ionization (ESI) after derivatization in a multisteroid assay [16]. To minimize sample preparation protocols by eliminating the derivatization step, hence the risks of poor recovery or interferences, ESI with its derivatization protocol which changes the analyte polarity to make it more ionizable, has been replaced by direct APPI ionization of the underivatized P_4 molecule. APPI is widely recognized as an efficient ionization method for less polar molecules, such as the steroid hormones [11,12]. This LC-MS/MS method with APPI ionization is currently being developed by the authors for multisteroid quantitation of underivatized sex steroid hormones, such as estradiol, estrone, estriol, and testosterone.

Quantitation of P_4 and other steroids by LC-MS/MS in human biological fluids is also usually performed using solid phase extraction (SPE [24,25]). Also, to reduce costs and increase the analytical speed, in view of the large number of samples normally investigated in animal studies, SPE extraction was substituted by simple liquid extraction, with no substantial loss in efficiency.

In the present study, quantitation results of plasma P_4 using LC-MS/MS confirmed similar hormonal kinetics for the three IPRD. Relatively greater P_4 concentrations were maintained for approximately 24 h as expected from the reduced levels of endogenous metabolizing enzymes that are specific to progesterone in OVX cows [26]. This peak was followed by a progressive decrease in P_4 concentration and an apparent steady-state from 96 to 192 h, where the released P_4 rate was similar to the metabolizing rates. When LC-MS/MS results were compared with those from RIA, great similarity was observed ($P < 0.05$). For more complex endocrine models, however, divergences are likely. Due to its superior selectivity, it is expected that

LC-MS/MS will provide more accurate and precise results. In the present study, one of the aims was to determine whether similar RIA and LC-MS/MS results would be attained. To minimize the possibility of cross-reactivity by interfering steroids, which is critical for RIA performance, ovariectomized cows were selected for this first comparative evaluation.

The emergence of LC-MS/MS as a gold standard analytical technique in veterinary reproductive science should be advanced and economic feasibility may involve multiuser projects due to the equipment costs and high-throughput use, as well as the use of multihormone assays to reach advantageous costs over the use of various RIA kits. The late emergence of LC-MS/MS in veterinary endocrinology may be primarily due to the lack of skilled personnel, especially those trained in method development, and the lack of LC-MS/MS facilities able to operate in veterinary endocrinology. Based on the present results, however, the merits of the LC-MS/MS system for steroid hormone quantitation appeared very attractive. The LC-APPI-MS/MS method proposed herein simplifies sample preparation (liquid extraction instead of SPE extraction) and eliminates the need of derivatization (which is mandatory for ESI). The method is therefore faster and simpler and displays high sensitivity, selectivity, and accuracy. In addition, when compared with RIA, it uses no radioactive materials, opening the possibility of developing robust, multihormone assays that may be applicable to many biological matrices, including plasma, saliva and milk, for which no specific RIA is commercially available.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.theriogenology.2011.05.033.

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Supplementary Table 1
Quantification values (ng/mL) for LC-MS/MS and RIA for the two animals receiving IPRD A.

Time (h)	Animal 1		Animal 2	
	LC-MS/MS	RIA	LC-MS/MS	RIA
0	0.84	0.67	1.23	0.88
2	4.59	6.08	6.76	6.34
4	5.04	6.57	8.65	8.92
6	5.62	6.59	8.47	8.26
8	5.14	6.08	7.95	8.19
10	5.29	5.89	7.46	7.81
12	4.97	5.73	8.82	9.32
24	4.48	4.89	6.58	7.61
48	3.89	3.83	5.76	5.90
72	3.56	3.64	5.58	5.81
96	3.33	3.41	4.13	4.82
120	3.06	2.82	3.54	3.81
144	2.78	2.78	4.40	4.01
168	3.62	3.97	3.40	3.03
192	3.98	2.91	2.89	2.80

There was no significant difference between method and animal.
LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; RIA, radioimmunoassay.

Supplementary Table 2
Quantification values (ng/mL) for LC-MS/MS and RIA for the two animals receiving IPRD B.

Time (h)	Animal 1		Animal 2	
	LC-MS/MS	RIA	LC-MS/MS	RIA
0	1.29	1.87	0.90	1.05
2	NA	6.97	5.59	6.40
4	6.37	7.33	5.85	6.59
6	6.41	7.18	6.32	6.99
8	6.79	7.47	5.58	7.05
10	5.92	6.33	6.11	5.79
12	5.18	7.16	5.67	5.66
24	4.09	6.01	4.64	5.36
48	4.06	6.07	3.51	4.01
72	NA	4.35	3.08	3.29
96	3.57	4.20	2.85	3.35
120	2.88	3.70	3.05	3.19
144	2.42	3.63	3.03	3.84
168	2.84	3.03	2.60	3.54
192	1.77	1.73	2.05	2.47

IPRD, intravaginal progesterone-releasing devices; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; NA, not available; RIA, radioimmunoassay.

Supplementary Table 3
Quantification values (ng/mL) for LC-MS/MS and RIA for the two animals receiving IPRD C.

Time (h)	Animal 1		Animal 2	
	LC-MS/MS	RIA	LC-MS/MS	RIA
0	0.53	0.38	1.41	0.76
2	5.27	6.05	6.33	7.13
4	5.02	5.54	6.80	8.10
6	4.79	5.75	6.89	7.85
8	4.87	5.05	6.82	7.84
10	4.87	4.44	6.12	7.98
12	4.15	4.76	6.08	6.70
24	4.22	4.44	5.23	5.34
48	3.40	3.89	4.82	5.29
72	NA	2.73	3.58	3.49
96	2.66	2.81	3.59	3.22
120	2.46	2.72	2.48	2.50
144	1.86	3.76	2.66	2.28
168	1.86	2.01	2.14	2.11
192	1.55	1.84	2.18	1.88

IPRD, intravaginal progesterone-releasing devices; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; NA, not available; RIA, radioimmunoassay.