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Development of a high-performance liquid chromatography–tandem mass spectrometry method for the determination of flurogestone acetate in ovine plasma

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Abstract

Flurogestone (FGA) is a synthetic progesterone, with a progestational action higher than that of progesterone itself. It is intended for vaginal use in large animals to induce oestrus synchronization. A quantitative method for the analysis of flurogestone acetate (FGA) in ovine plasma by high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) has been developed. After the incorporation of megestrol acetate (MGA) as internal standard (IS) and followed by a liquid–liquid extraction from plasma, FGA and MGA were chromatographed using a reverse-phase HPLC column and detected by tandem mass spectrometry with a TurboIonSpray® source. Multiple reaction-monitoring (MRM) mode was used for the quantitative determination of FGA in ovine plasma. The precursor ions $[M+H]^+$ at *m/z* 407.2 and 385.1 for FGA and MGA, respectively, produced product ions at *m/z* 267.1/285.1 for FGA and *m/z* 267.1/224.0 for MGA. The validated concentration range was 0.2–5.0 ng/ml based on 500 μl plasma aliquots. The lower limit of quantitation was 0.2 ng/ml. Fully validated selectivity, accuracy, precision and reproducibility criteria for routine use in pharmacokinetic studies were demonstrated.

Keywords: Oestrus synchronization; Flurogestone acetate

1. Introduction

Flurogestone acetate (FGA) (17α-acetoxy-9α-fluro-11β-hydroxy-pregn-4-ene-3,20-dione) is a potent synthetic progestagen that has been used for more than 40 years in veterinary medicine for oestrus synchronization in sheep and goat [1]. The objective of oestrus synchronization is to make ewes fertilizable at a predetermined time schedule without oestrus detection, reducing the time spent on animal handling [2,3]. The synchronization of oestrus in ewes is achieved by the continuous administration of natural progesterone, or synthetic progestins such as FGA, during 14 days, resulting in the inhibition of the hypothalamic–hypophyseal axis, plus an intramuscular injection of pregnant mare serum gonadotrophin (PMSG) at the end of the treatment. Two to four days later, treated ewes re-enter oestrus and ovulate, allowing planned artificial insemination and breeding. The first FGA sustained delivery system was developed by Robinson et al. and consisted of a polyurethane vaginal sponge [4]. To date, none of the available FGA delivery system for oestrus synchronization in ewes has been biodegradable. Therefore, experiments are currently in progress in our department in order to develop an injectable, biodegradable FGA delivery system [5]. Over the years, few studies were carried out to describe the metabolism and the pharmacokinetic profile of flurogestone acetate. An in vitro metabolism study with ovine hepatocyte showed than flurogestone acetate was metabolised to a number of hydroxylated products, indicating that flurogestone acetate follows the normal breakdown pathway for progestagens [6]. In vivo, tissue residue studies showed that flurogestone acetate was recovered intact until 5 days post-dosing.

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The concentrations of FGA in biological fluids are around the ng/ml level or even lower, therefore a more rapid and sensitive method than radioimmunoassay (RIA) [7] was required for high throughput analysis. The recent improvements in mass spectrometry, such as electrospray ionisation (ESI) and atmospheric-pressure chemical ionisation (APCI), allowed enhanced sensitivity, increased selectivity and the robustness of these methods [8–10].

Therefore, high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) is currently recognized as a powerful tool to analyse biological samples.

In this paper, a high-performance liquid chromatography–tandem mass spectrometry method for routine determination of FGA in ovine plasma was developed using megestrol acetate (MGA) as internal standard (IS).

2. Materials and methods

2.1. Materials

The molecular mass of flurogestone acetate is 406.49 g/mol. All plasma concentrations, stock solutions and dilutions are given in mass units. The flurogestone acetate (Fig. 1; purity: 99.92%) was used for preparation of stock solutions. Megestrol acetate was used (purity: 100.34%) as internal standard. Both standards were obtained from CEVA Santé Animale (Libourne, France).

Methanol, ethyl acetate and ammonium formate were of analytical grade and purchased from Merck (Darmstadt, Germany), Prolabo (Fontenay sous-bois, France) and Carlo Erba (Val de Reuil, France), respectively. Distilled water was purified using a Milli-Q system (Milford, MA, USA).

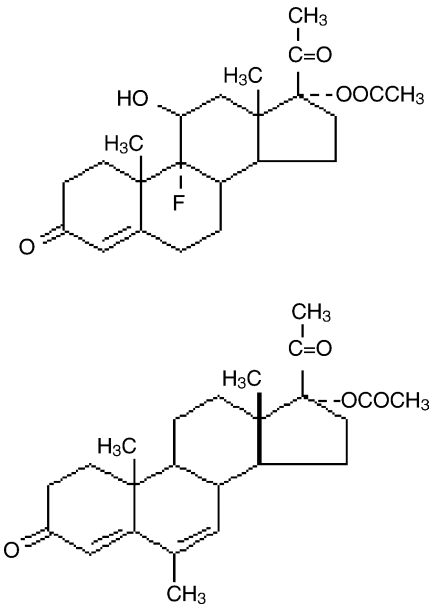

Fig. 1. Structure of flurogestone acetate (FGA) and its internal standard megestrol acetate (MGA).

2.2. Preparation of stock solutions and standards

Stock standard solutions of FGA and MGA (100 μg/ml) were prepared by dissolving the compound in methanol. These stock standard solutions were used to make calibration standards and QC samples. Eight calibration standards solutions at concentrations 5, 10, 25, 50, 75, 100 and 125 ng/ml for FGA, four validation standards at 5, 10, 62.5 and 106.25 ng/ml and a solution at 50 ng/ml for MGA were prepared by diluting the appropriate stock standard solutions with water. Next, all calibration and validation solutions were used to prepared plasma calibration standards from 0.2 to 5 ng/ml and QC samples containing 0.2 ng/ml (LLOQ), 0.4 ng/ml (low), 2.5 ng/ml (medium), and 4.25 ng/ml (high) of FGA.

2.3. Sample preparation procedure

Blood samples were collected from Lacaune ewes by jugular venipuncture. Aliquots of 500 μl of plasma samples were transferred into polypropylene tubes and 20 μl of the working solution of IS MGA (50 ng/ml) were added. The samples were vortexed. Next, 2 ml of ethyl acetate were added and samples were placed on a linear shaker for 10 min and centrifuged at 2000 × *g* during 5 min. The ethyl acetate phases were transferred into glass tubes and evaporated to dryness under a nitrogen stream at 40 °C. The residues were reconstituted in 200 μl of 5 mM ammonium formate/methanol (30/70, v/v). The samples were sonicated for 2 min, vortexed and centrifuged at 2000 × *g* for 10 min. Fifty microliters of supernatant were injected into the HPLC–MS/MS system.

2.4. Chromatographic conditions

Separation was carried out at 35 °C using a Kromasil C4 column (15 cm × 3.0 mm) packed with 5 μm particle size (Bios Analytical, L'Union, France). The chromatographic system consisted of a G1312A pump, a G1313A auto-sampler and a G1316A oven all from Agilent (Waldbronn, Germany). The mobile phase consisted of methanol/5 mM ammonium formate buffer (pH 6.2). Different combinations of 5 mM ammonium formate and methanol in the mobile phase were investigated in order to obtain an optimal mobile phase. The final mixture used was that giving the minimum of sodium adducts. The mobile phase was delivered under a gradient elution (i.e. from $t = 0$ to $t = 7$ min, the mixture consisted of 30% ammonium formate 5 mM and 70% methanol; it changed linearly in 0.2 min to 5% ammonium formate 5 mM and 95% methanol). The system remained stable during 3 min and returned to its initial state in 0.3 min. The flow rate was 0.4 ml/min. The retention times were 4.5 min for FGA and 6.6 min for MGA.

2.5. Mass spectrometry

A Perkin-Elmer Sciex API 3000 (Perkin-Elmer, Foster City, CA, USA) equipped with a TurboIonSpray® interface

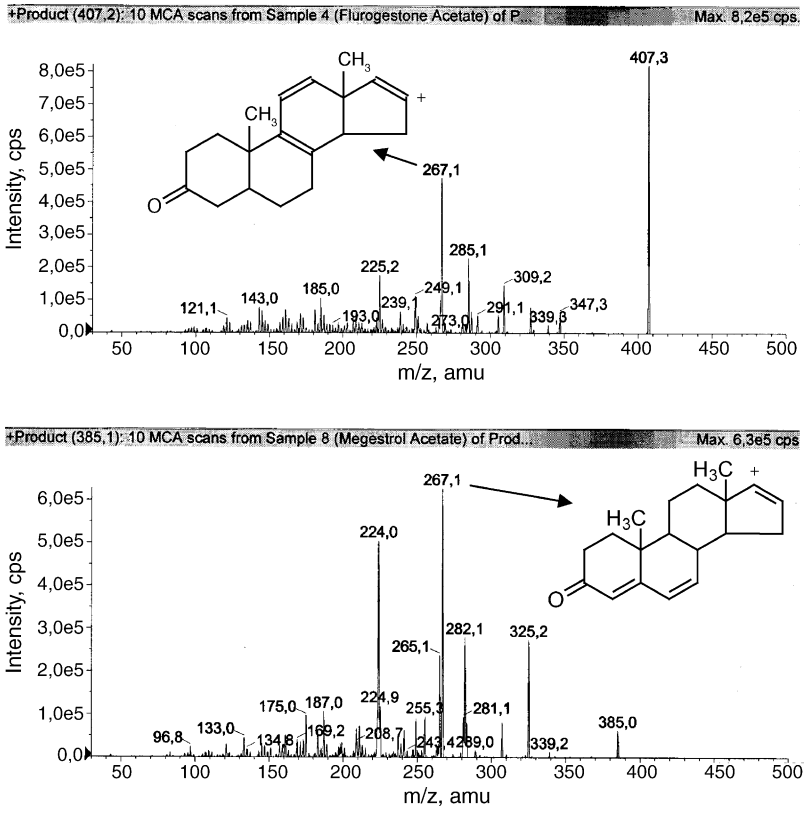

Fig. 2. Product ion mass spectra for FGA and MGA (internal standard) under the MS/MS conditions and expected fragmentation.

was used as a detector. It was operated in positive MRM mode having precursor ion at *m/z* 407.2 (FGA) and *m/z* 385.1 (MGA), and product ions of *m/z* 267.1 (FGA and MGA), suggested product ion scans shown in Fig. 2, respectively. The declustering potential was 73 V, focusing potential was 350 V, entrance potential was −13 V, collision energy was 31, ion spray voltage was 5700 V, and probe temperature was 475 °C.

Instrumental conditions for the MRM transition of the analyte were optimised using Analyst version 1.1 software. Mass spectrometry conditions were optimised using a syringe pump infusion (5 μl/min.). Infusion of FGA and internal standard were done in the mobile phase. Ammonium formate was choice in the place of the formic acid in order to decrease sodium adducts (Fig. 3). High-flow gas parameters (curtain, nebuliser and heater gas) were optimised by making successive flow injections, while introducing mobile phase into the ionisation source at 400 μl/min.

2.6. Assay validation

To evaluate the extraction procedure recovery described earlier, three batches of drug-free ovine plasma samples were spiked with known amounts of FGA and compared to the FGA working solution. The matrix effect was determined by comparing blank matrix samples from three different individuals spiked within the range 0.2 ng/ml (LLOQ) to 5.0 ng/ml (ULOQ) of FGA. The response function of the assay within the range 0.2 ng/ml (LLOQ) to 5.0 ng/ml (ULOQ) was verified by plotting the chromatographic peaks area ratios of analyte to internal standard versus nominal concentration using the appropriate weighing factor. The linearity range of the assay was validated by means of coefficient of determination and statistical goodness of fit (i.e. power model) [11].

In order to assess the stability of the extracts during the analytical run, plasma samples (QC samples) spiked with FGA (0.2, 0.4, 2.5 and 4.25 ng/ml) were submitted to the extraction procedure and injected during 24 h (11 injections per concentration level). The measured responses of each concentration level were compared to the measured response of the first standard injection.

The intra-assay was determined by repeated analyses ($n = 4$) on the same day of the following QC samples: 0.2, 0.4, 2.5 and 4.25 ng/ml. Whereas, the inter-assay precision and accuracy were determined by repeated analysing ($n = 12$) for three consecutive days of the following working standard solutions for validation: 0.2, 0.4, 2.5 and 4.25 ng/ml.

3. Results and discussion

3.1. Quantitative analysis

The predominant ions were the protonated molecular ions $[M + H]^+$ with *m/z* values of 407.2 and 385.1 for FGA

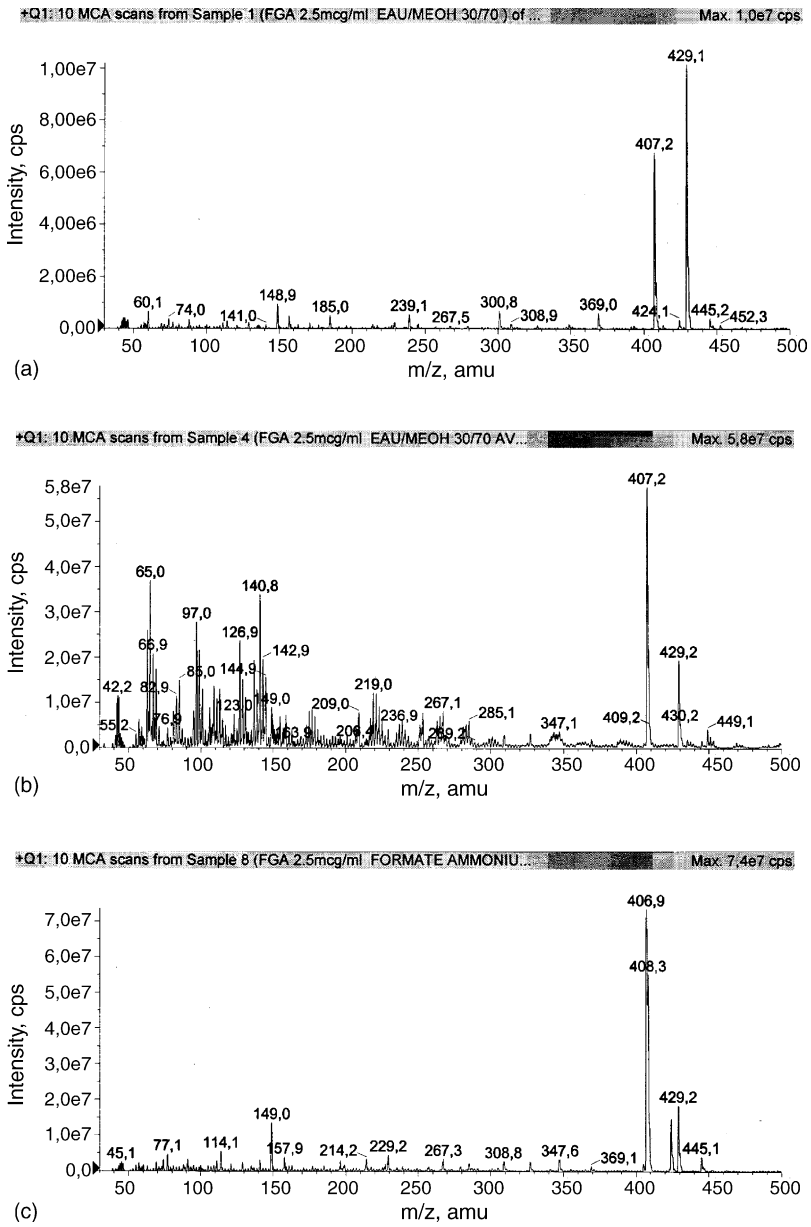

Fig. 3. MS scans of FGA in several mobile phases: (a) methanol/water (70/30); (b) methanol/water (70/30) 0.1% formic acid; (c) methanol/5 mM ammonium formate (70/30).

and MGA, respectively. These positive molecular ions were therefore used as the precursor ions in the MS/MS experiment. Multiple reaction-monitoring (MRM) mode was used for the quantitative determination of FGA and MGA in ovine plasma. Using this mode, ions that are not related to the target compound are filtered out, thus minimizing the potential influence of the endogenous compound from the matrix sample. Therefore, selection and optimisation of product ions was of great importance in obtaining the best selectivity and sensitivity. Transitions ions at *m/z* 407.2 → 267.1 for FGA and *m/z* 385.1 → 267.1 for MGA allowed sensitive and selective detection of the two compounds.

3.2. Matrix effect, response function, linearity, stability, precision, sensitivity and recovery

The detection limit (LOD) was calculated on 20 representative blank samples. LOD was considered to be equal to the value of the mean plus three times the standard deviation (0.036 ng/ml ± 3 × 0.07). The limit of detection was set at 0.058 ng/ml. The lower limit of quantitation (LLOQ) on the calibration curve was accepted as the limit of quantitation if the following conditions are met; the analyte response at the LLOQ was the blank response plus six times the standard deviation (0.036 ng/ml + 6 × 0.07); as the analyte response was reproducible with a precision of 20% and

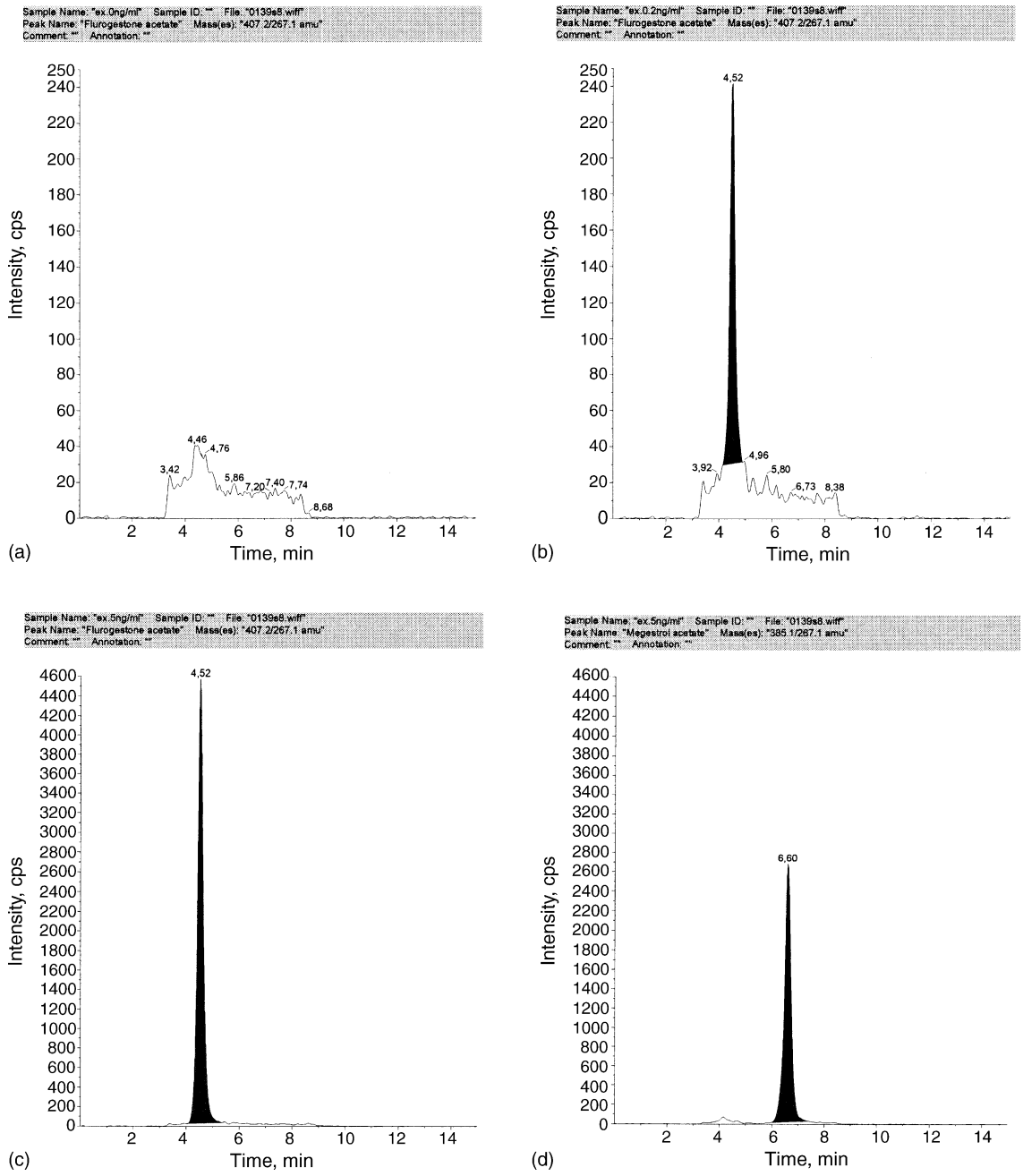

Fig. 4. Chromatograms of blank plasma (a), plasma spiked with 0.2 ng/ml of FGA (LLOQ) (b), 5 ng/ml of FGA (HLOQ) (c) and corresponding internal standard (1 ng/ml) (d).

accuracy within ±20%. The lower limit of quantitation was set at and 0.2 ng/ml. A typical blank chromatogram is shown in Fig. 4a.

In order to assess the stability of the extracts during the analytical run, plasma samples spiked with FGA (0.2, 0.4, 2.5 and 4.25 ng/ml) were submitted to the extraction procedure and injected during 24 h (11 injections per level). The deviations were within the interval of ±20% (−10.6% for 0.2 ng/ml and 5.6% for 2.5 ng/ml).

During LC–MS/MS analysis, it is possible for components of the matrix which remain after the extraction to either suppress or enhance the signal in the mass spectrometer. The matrix effect calculated for all three blank samples was within 11.9% of the spiked value. These data demonstrated the weak of impact of matrix suppression. The recovery of the analyte was assessed by comparing the peak areas of analyte with those of equivalent amounts of reference standard of analyte. The three replicates of seven concentrations of analyte (concentration levels of the calibration curve) were determined and the recovery was ranged between 72.8 and 82.3% (Table 1).

Table 1
Recovery rate of FGA plasma samples

Nominal amount (ng/ml)	Mean area with extraction	Mean area without extraction	Recovery rate (%)
0.2	1922.7	2642.5	72.8
0.4	4018.9	5399.0	74.4
1	11490.8	14794.0	77.7
2	23888.7	30015.2	79.6
3	37271.3	46336.8	80.4
4	50429.1	61273.4	82.3
5	60911.7	79248.8	76.9

Table 2
Calibration graph data

Batch ID	Parameters		
	Slope	*Y*-intercept	*r*
0139/1	0.436	−0.0115	0.9962
0139/2	0.403	−0.0194	0.9948
0139/3	0.376	0.0123	0.9926
0139/4	0.344	0.0255	0.9956
0139/5	0.316	0.0010	0.9966
Mean	0.375	0.00158	0.9952
SD	0.047	0.01802	0.0016
C.V. (%)	12.61	–	0.16

Table 3
Intra- and inter-assay precision and accuracy of FGA plasma samples

Nominal amount (ng/ml)	Within-days		Inter-days	
	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)
0.2	3.52	9.5	13.14	−6.7
0.4	4.53	5.6	12.19	−7.0
2.5	2.56	5.91	10.75	−4.4
4.25	3.66	12.5	12.01	−0.3

The calibration type of first order with weighing factor ($1/x^2$) was selected as "the best fit" straight line through the calibration range (0.2–5.0 ng/ml). Based on the regression coefficients (r of 0.9952 in mean) for all calibration curves (Table 2), and according to the goodness of fit, the assay was considered linear within the plasma calibration range.

The data of intra-assay precision and accuracy were obtained from analysis of QC samples of 0.2, 0.4, 2.5 and 4.25 ng/ml in a single day ($n = 4$) (Table 2). The inter-assay precision and accuracy ($n = 12$) were determined by analysing quality control samples of 0.2, 0.4, 2.5 and 4.25 ng/ml on three different days and results are reported in Table 2. The intra-assay precision was below 5% for each concentration level. The inter-assay precision was below 15% (Table 3). The accuracy was below 12.5%. These data confirm the good precision of the method. Representative MRM chromatograms of plasma are presented in Fig. 4.

Stability of the extract after freeze–thaw cycles was tested by replicate analyses ($n = 3$) of blank ewe plasma spiked at two concentrations levels (0.4 and 4.25 μg/l) by submitting them to three freeze–thaw cycles. Samples were stables after three cycles. Samples were also stable after 6 months of conservation at −80 °C.

4. Conclusion

An HPLC–MS/MS method was developed and validated for the routine determination of FGA in ovine plasma. The assay was shown to be specific, accurate, precise and reproducible. The absence of matrix effect was also demonstrated. Results have shown that the described method is more suitable for high throughput clinical analysis. Therefore, the method is currently used in pharmacokinetic studies for the development of an injectable and biodegradable FGA-controlled delivery system for oestrus synchronization in ewes.

Acknowledgement

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