Development and application of an UHPLC-MS/MS method for the simultaneous determination of 17 steroidal hormones in equine serum

Short title

Quantification of steroidal hormones in equine serum by HPLC-MS/MS

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Abstract

A new, fast and simple analytical method that is able to identify and quantify simultaneously 17 steroid hormones and metabolites (Pregnenolone, 17-OH-Pregnenolone, Progesterone, 17-OH Progesterone, Androsterone, Androstenedione, DHEA, DHEAS, Testosterone, Cortisol, Corticosterone, Aldosterone, 11-Deoxycortisol, 11-Deoxycorticosterone, Dihydrotestosterone, Estrone, Estradiol) has been developed in equine serum using the UHPLC-MS/MS technique. 400 µL of sample were deproteinized with 1000 µl of acetonitrile, evaporated, restored with 50 µl of a solution of 25% methanol and injected in UHPLC-MS/MS triple quadrupole. The recovery percentage obtained by spiking the matrix at two different concentrations with a standard mixture of steroid hormones was in all cases higher than 85.60 % and with the percentage of coefficient of variation (CV) lower than 8.37%. The range of the correlation coefficients of the calibration curves of the analyzed compounds was 0.9922–0.9986, and the limits of detection (LODs) and limits of quantification (LOQs) were in the range of 0.002–2 ng ml^{-1} and 0.0055-5.5 ng ml^{-1}, respectively. The detected LOQ for testosterone (i.e. 50 pg ml^{-1}) is two-fold lower with respect to its threshold admitted in geldings plasma (100 pg ml^{-1} free testosterone). The high sensitivity and the quantitative aspect of the method permitted to detect most of steroids in equine serum. Once validated, the method was used to quantify 17 steroid hormones in mare, stallion and gelding serum samples. The main steroids detected were corticosterone (range 37.25-51.26 ng ml^{-1}) and cortisol (range 32.57-52.24 ng ml^{-1}), followed by 17-OH-pregnenolone, dihydrotestosterone and pregnenolone.

Keywords

UHPLC-MS/MS, steroidal hormones, equine serum, hormones, validation

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Introduction

Steroids are a large class of compounds deriving from cholesterol that play a critical role in transmitting a vast array of biological signals in the organism.[1] Their functions can be broadly grouped into several categories: reproduction and sexual differentiation, development and growth, maintenance of the internal environment, and regulation of metabolism and nutrient supply.[1] Doping control in equine sports poses different challenges compared to those in human sports. In the latter, doping offences are committed almost without exception to improve performance. In equine sports, both performance enhancing and performance impairing substances (or methods) may be used in order to manipulate the outcome of the competition. This may be more predominant in horse racing where the potential gain from betting on other horses may outweigh the prize money from winning.[2] At present, there are only 11 compounds in the list 6A of prohibited substances with international thresholds in either urine or plasma or both.[3] Apart from carbon dioxide, dimethyl sulphoxide, salicylic acid and theobromine, testosterone is still the only steroids regulated in plasma and its threshold in plasma geldings is 100 pg ml^{-1} (free testosterone).[3] Nevertheless, steroids, are highly used in order to improve the performances or hiding some health conditions of the animal, before or after a race, or during a horse trade. These substances are not harmless, because they may cause some pathologies like lung bleeding, hepatotoxicity, cardiac hypertrophy, tendinitis and articulation problems, cancer, which could possibly lead to stroke and death.[4]

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Quantitative assessment of steroidal hormones and their metabolites is relevant to the diagnosis and treatment of a variety of diseases and conditions, including disorders of puberty, amenorrhea, infertility, polycystic ovary syndrome, osteoporosis, adrenal insufficiency, hypogonadism, cognitive dysfunction, cardiovascular diseases and hormone-related malignancies.[1] For the time being, many analytical procedures existing in literature for steroids are self-made analysis, often too complex or too long and expensive to be replicated in external clinical laboratories**.** Moreover, these methods are often based on immunoassay analysis, with poor sensitivity and high possibility of false negative and false positive response.[5-9] Huang et al. (2008) developed a method to determine six sexual steroid hormones in urine matrix by stir bar sorptive extraction (SBSE) coupled to high performance liquid chromatography (HPLC)-diode array detector (DAD);[10] Magnisali et al (2008) used GC-MS (gas chromatography-mass spectrometry) for analyzing six steroids in serum of neonates;[11] Caron et al. (2015) reported a gas chromatography–tandem mass spectrometry (GC-MS/MS) method for the simultaneous quantification of ten endogenous steroids in serum from men, premenopausal and postmenopausal women.[1] From literature, clearly arise that the majority of methods for steroids analysis focused firstly on human serum, and secondly on the concomitant help of HPLC-MS/MS (high performance liquid chromatography-tandem mass spectrometry), that is the ideal technique due to the high specificity and sensitivity. For example, Buttler et al. (2015) described the simultaneous measurement of 3 steroids, namely testosterone, androstenedione, dehydroepiandrosterone (DHEA) using ID (isotope dilution)-LC-MS/MS;[12] Ray et al. (2015) used LC-MS/MS combined with ion mobility spectrometry for the analysis of 5 endogenous steroids;[13] Ke et al. (2014) analyzed seven steroidal compounds by using UHPLC-MS/MS (with Q-trap like mass analyzer);[14] Peitzch et al. (2015) analyzed simultaneously 15 adrenal steroids in LC-MS/MS;[15] in all cases the matrix was human serum. On the contrary, very few methods

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describing the simultaneous quantification of steroid hormones in equine serum have been reported in literature. Guan et al. (2005), analyzed eight major anabolic steroids in equine plasma[16] whereas Kaabia et al. (2013) analyzed a high number (20) of steroid esters in bovine and equine serum and plasma, following a long and time consuming procedure, i.e. solid phase extraction (SPE) before analysis with UHPLC–ESI-MS/MS.[17] Because of that, it is crucial to develop and validate a new, fast, and simple analytical procedure that can quantify ~~as more as~~ many steroids ~~as possible~~, with high sensitivity and reproducibility, in a single and short run providing reliable results transferable to external veterinary laboratories. Thus, the aim of our work was to set-up a new UHPLC-MS/MS triple quadrupole method to detect and quantify seventeen hormones and metabolites in equine serum. Quantifiable hormones with the proposed method are: Pregnenolone, 17-OH-Pregnenolone, Progesterone, 17-OH-Progesterone, Androsterone, Androstenedione, DHEA, DHEAS, Testosterone, Cortisol, Corticosterone, Aldosterone, 11-Deoxycortisol, 11-Deoxycorticosterone, Dihydrotestosterone, Estrone, Estradiol. Three deuterated hormones (Cortisol-D4, Aldosterone-D7, Testosterone-D3) have been used as internal standards in order to set a more accurate and precise procedure (**Table 1**). Most of the compounds chosen in the current method are often different from those reported in literature and not usually included in analytical methods.[16, 17] The procedure is fast, sample preparation is easy, the method is sensitive, accurate and robust and it could bring a remarkable saving of time and money with respect to previously reported methods. After full method validation, this procedure has been successfully applied to the analysis of serum samples from different kind of horses (mare, stallion and gelding).

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Experimental

Materials and standards

Steroids (purity >99%) d_3-Testosterone, d_4 Cortisol and d_7-Aldosterone were purchased from Sigma-Aldrich (Milano, Italy). Individual stock solution of steroid hormones and metabolites were prepared by dissolving 1 mg of each compound in 1 ml of HPLC-grade methanol. The standard working solutions were obtained by diluting the stock solution at the concentration needed with methanol. HPLC-grade acetonitrile and methanol were purchased from Carlo Erba (Milano, Italy). HPLC-grade formic acid (99 %) was obtained from Merck (Darmstadt, Germany). Deionized water (>18MΩ cm resistivity) was purified using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All solvents and solutions were filtered through a 0.2 μm nylon membrane filter from Minisart RC 4, Sartorium Stedim (Goettingen, Germany) before transferring them into injection vials. Sterile glass tubes for blood collection were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA).

Sample collection

The entire equine blood was collected from male and female healthy horses provided by the university veterinary hospital in Matelica (MC). The blood was allowed to clot and centrifuged to obtain serum. An aliquot of serum from each horse was refrigerated at -20°C and stored to be used as real sample; the rest of serum was purified using charcoal in order to obtain a matrix free of any hormone.

Analytical procedure

All analytical procedures were performed in polypropylene vials, test tubes and plastic centrifuge tubes in order to preserve the concentration and stability of the hormones. The glass has demonstrated that it could interfere with those molecules (*Data not shown*).

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Preparation of Steroids free serum

50 mL of serum and 1g of charcoal were stirred under magnetic agitation overnight in order to create a steroid free serum used in all validation steps (except for recovery studies and matrix effect performed in normal serum), as reported by Magnisali et al.[11] The solution was centrifuged at 4000 rpm for 20 minutes in plastic test tube and the supernatant was centrifuged two more times at 14000 rpm for 20 minutes in plastic centrifuge tubes in order to clean the serum from the charcoal residues. After these steps, the serum appears light yellow and clear. The purified serum was stored at 4°C and used to set-up the method and perform full validation (except for recovery studies and matrix effect, performed in normal serum).

Sample preparation

400 µL of serum (normal or steroids free) were transferred into a plastic centrifuge tubes, deproteinized using 1 ml of deproteinizing solution (acetonitrile) and vortexed for 1 minute. The deproteinized solution was centrifugated at 14000 rpm for 15 minutes, then the supernatant is transferred into polypropylene test tube.

Evaporation process

The supernatant was evaporated using nitrogen gas flow. The dried samples were restored using 50 µl of 25% methanol, transferred in plastic centrifuge tubes and centrifuged at 14000 rpm for 15 minutes. The supernatant was transferred in high recovery vials and injected in the UHPLC-ESI-MS/MS system.

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Liquid chromatography tandem mass spectrometry

UHPLC-ESI-MS/MS studies were performed using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an ESI source operating in negative and positive ionization mode. The separation of analytes was achieved on a Zorbax RRHD C18 analytical column (50 x 2.10 mm i.d., 1.8 μm) from Agilent (USA). The mobile phase for UHPLC-MS/MS analyses was a mixture of water (A) and acetonitrile (B) both containing 0.1% formic acid at 0.6 ml/min with a gradient elution: 0 min 15 % B, 3.2 min 42% B, 4.5 min 42 % B, 7 min 90 %B, 9 min 90 % B, 9.50 min. 15% B, and kept at 15 % B until the end of the run (11 min). The injection volume was 5 μl, performed with auto-sampler. The temperature of the column was 10 °C and the temperature of the drying gas in the ionization source was 300 °C. The gas flow was 12 l/min, the nebulizer pressure was 40 psi and the capillary voltage was 4000 V (negative and positive). Detection was performed in the "multiple reaction monitoring" (MRM) mode dividing the run time in seven segments as reported in **Table 1**. The most abundant product ion was used for quantification, and the rest of the product ions were used for qualification. The monitored compounds, abbreviation, selected ion transition and the settings of the mass analyzer are reported in **Table 1**.

Calibration standards and controls

In order to validate the method, different concentrations were used, starting from the LOQ (lowest one), to C1 (low concentration), CM (Medium concentration), C2 (high concentration), CU (upper concentration) (low range standard curve, 5 points) and four additional "upper" concentrations (U1-U2-U3-U4) for high range standard curve, for a total of 9 points. **Table 2** reported the value of all the concentrations chosen for the calibration curve and the validation steps for the different compounds.

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Internal Standard

Three deuterated internal standards were used in order to increase the robustness of the method. The standards were added before deproteinization step at the concentration of: 250 ng/ml d_7–aldosterone, 100 ng/ml d_3-testosterone, 1000 ng/ml d_4–cortisol. D_3-testosterone was used as internal standard for TESTO, ANDD, DHEAS, DHEA, ESTRO, ANDRO, DHT and ESTRA, d_4–cortisol for 11-DOC, 17-OH-PROG, PRE, CORT, 17-OH-PRE, d_7–aldosterone for ALDO, CoCo, PRO, 11-DCC.

Results and Discussion

Optimization of chromatographic conditions

Different solvents were tested and the mixture of water-formic acid 0.1%, (mobile phase A) and acetonitrile-formic acid 0.1% (mobile phase B) was the ideal combination for the analysis and separation of this complex mixture of standards. Use of methanol as mobile phase B, or only water as mobile phase A, leads to worse separation among peaks and a lower sensitivity. Moreover, considering the different and wide degrees of polarity of the seventeen steroids, the gradient elution was used to achieve the best separation. Under the optimum gradient conditions, as reported in paragraph ~~2.7.1~~ "Liquid chromatography tandem mass spectrometry", the baseline separation of all peaks of those compounds was achieved. In our experiments we found that this time of final conditioning (at least 1.5 min.) is indispensable for reproducibility of retention times of the monitored analytes. However, other gradient conditions ~~, (low or fast and more extreme gradient)~~ caused a poor separation of some peaks or overlapping of analytes. Additionally, the flow rate at 0.6 ml min^{-1} seems to be the best for our purpose, instead flow rate of 0.4 and 0.5 ml min^{-1} caused a longer analytical time without improvement, and flow rate of 0.7 ml min^{-1} caused a worst separation of all steroids.

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Mass spectrometry conditions and ESI (+) and ESI (-) MS spectra

Mobile phase composition and additives may have a significant influence on the response of the solute with ESI source. Thus, two different mobile phase compositions (i.e. acetronitrile–water and methanol–water both containing formic acid) were tested and the first one was chosen for the analysis, as the response was significantly higher than that using methanol–water and also chromatography separation and resolution of peaks were excellent. Even using an aprotic organic solvent plus water, the mobile phases provided enough protons in positive mode sufficient for steroidal hormones protonation without affecting the abundance of ions formed.[18] According to literature, the use of formic acid as additives is often a good choice in positive mode to increase the response of target compounds[18] and, in fact, in our case it improved both ionization and chromatographic separation/resolution of peaks. For most of the monitored compounds, the precursor ion was the protonated molecule $[M+H]^+$ in positive polarity and the deprotonated molecule $[M-H]^-$ for the only analyte (DHEAS) monitored in negative polarity. On the other hand, only for ESTRA, DHT and PRE, the precursor ion in positive polarity was the $[M+H-H_20]^+$ as reported by Shao et al.[18] Abundant $[M+H]^+$ or $[M-H]^-$ ions are always desired for sensitive, qualitative and quantitative methods. Conversely, pseudo-molecular ions (in particular solvent adduct) are undesirable since they decrease abundance of $[M+H]^+$ ions. Even if methanol is, for this aspect, prefeared over acetonitrile as it showed lower proton affinity, and thus it has lower tendency to form solvent adduct ions,[16] we similarly obtained selective ionization using acetonitrile.

The tandem MS product ion spectrum in the triple quadrupole mass spectrometer is the result of a one-step process in which several product ions are formed and in which it is difficult to ascertain the relationship of the precursor and product ions. Compared with the fragmentation in other mass analyzers (ion trap, Orbitrap, Q-TOF), a much higher abundance of product ions formed by recyclization cleavages is found in the product ion spectra in the triple

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quadrupole. This occurs for 8 steroids, whose quantitative product ion formed is not easily decipherable with a low resolution mass analyzer like triple quadrupole. On the other side, for ALDO, CoCo, 17-OH PROG, DHEA, ESTRO and ANDRO, the quantitative product ion is the $[M–H_20+H]^+$ and for ESTRA, DHT and PRE the main product ion formed was the $[M–2H_20+H]^+$, as reported in literature.[19]

Method validation

The analytical characteristics of the developed method were investigated, including linearity, limit of detection (LODs), limits of quantification (LOQs), accuracy and precision, recovery and matrix effect, to evaluate their efficacy for application on the analysis of steroids hormones in equine samples.

Concentration values used for the validation steps have been chosen according to the LOQ of each compound (**Table 2**).

Linearity

As mentioned before, two types of standard curves were prepared by analyzing spiked hormones free serum samples (see ~~2.4~~ paragraph "Calibration standards and controls") at different concentration levels. The calibration curves were calculated with all the concentrations from the LOQ to the CU, (low range standard curve, 5 points) form data obtained during a 3-day validation and using three repetitions of each value every day. The obtained R^2 are higher than 0.9990 % (*Data not shown*).

The high range standard curve was calculated with all the nine concentrations from the LOQ to the U4, in order to test the upper limit of linearity and to assess linearity in a wider dynamic range. The data were obtained from three repetitions of all concentration, and the results were expressed in terms of coefficient of linear regression (R^2), slope and intercept of the seventeen curves obtained. The developed method displayed good linearity, being the

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correlation coefficients range of the analyzed compounds calibration curves in the range 0.9922–0.9986 (*Data not shown*).

The limits of detection (LODs) and limits of quantification (LOQs) found in the study were in the range of 0.002–2 ng ml^{-1} and 0.0055-5.5 ng ml^{-1}, respectively. The LOD and LOQ obtained in the present study are similar or even lower than the limits described in literature;[16, 17, 20] even if most of the compounds chosen in the current method are often different from those included in analytical method available in literature. Moreover, LOQ for testosterone is equal to 0.05 ng ml^{-1}, twice lower with respect to 0.1 ng ml^{-1} (or 100 pg ml^{-1}) that is the admitted limit in plasma geldings.[3]

Precision

Precision is the closeness of agreement between independent test results obtained under stipulated conditions. It is usually specified in terms of standard deviation or relative standard deviation.[21]

The precision (intra- and inter-day) was calculated with the LOQ, CM, CU and U3, from data obtained during a 3-day validation (**Table 3**). Each day, five repetitions of the three concentrations were tested and the precision results for each concentration were reported in term of CV% (coefficient of variation). The coefficient of variation for all compounds were satisfactory and within the range 0.33–18.74% (**Table 3**). The inter-day (n=5) method precision was also satisfactory as expressed by the percent RSD values that were obtained. At the LOQ concentration, the percent RSD values were within the range, 13.46–17.82%, at the CM concentration the% RSD values were 7.39–18.74%, at the CU concentration the % RSD values were 3.52–18.15% and at the U3 concentration the % RSD values were 0.33–9.21% for the 17 steroid hormones examined in this study (**Table 3**).

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Accuracy

Accuracy is the closeness of agreement between a test result and the accepted reference value of the property being measured.[21]

The accuracy (intra- and inter-day) was calculated using the C1, C2 and U3 spiked concentrations, from the data obtained during a 3-day validation. The results are reported in **Table 4**, and expressed in term of relative error %. The percentage relative errors for all the analytes were satisfactory and within the range, 0.92–13.90% (**Table 4**). The inter-day (n=5) method accuracy was also satisfactory as expressed by the percent RSD values that were obtained: at the C1 concentration, the % RE values were in the range 6.52–13.90% ~~and~~ at the C2 concentration, the % RE values were 2.26–7.72% and at the U3 concentration, the % RE values were 1.93–4.94%.

Recovery

Recovery studies were performed by spiking normal equine serum with a mixture standard of the seventeen hormones. The recovery value was obtained using the following formula: $((A_{se}-As_{blank})/A_{std})$ X 100, where A_{se} is the area about the serum enriched with a low concentration (C1 and CM) of all the compounds, A_{blank} is the area of analytes detected in the serum, A_{std} is the area of a mixture standard of all the compounds dissolved in methanol. The recoveries obtained by spiking the matrix at the CM concentration were in the range of 91.05-97.66%, with CV lower than 5.04% (**Table 5**). Moreover, the recoveries at a concentration of C1 were in the range 85.60-99.39%, with CV lower than 8.37% (**Table 5**).

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Matrix effect

Matrix effects can lead to either a reduced response (ion suppression) or an increased response (ion enhancement) of the mass spectrometry system.[21] These effects can severely compromise quantitative analysis of biological samples using LC-ESI-MS. In order to investigate the matrix effect, a post column infusion was performed. Post column infusion is one of the best techniques used to obtain qualitative informations about matrix effects.[22] A methanolic mixture of all the compounds at CM concentration have been infused in the ESI using a micro pump and then an injection of extracted serum was performed. As shown in **Figure 1,** the signal remains constant for almost all the chromatographic time, except for a "valley" (signal suppression) at 7.5 minutes. All the compounds have a retention time shorter than 7 minutes, thus it is evident that the matrix does not have any suppression or enhancement effect in these analysis.

Specificity

High specificity was achieved using tandem mass spectrometry. Both retention time stability and multiple precursor/product ion pairs were utilized to demonstrate the specificity of the method. Reproducibility of the chromatographic retention time for each compound was examined five times over a five day period (n=25). The retention times using this method were stable with RSD % values ≤ 0.98 %.

Specific precursor/product ion transitions were identified for each steroids and the MRM transition with the most abundant product ion was selected for quantitation and the other product ion was selected for qualification (**Table 1**).

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Application of the developed method to the analysis of equine serum.

The high sensitivity and the quantitative aspect of the method permitted to detect most of steroids in four equine serum, i.e. two mare, one stallion and one gelding serum.

Only aldosterone, DHEAS, DHEA and estrone were not detected in any samples (**Table 6**). The main steroids found in the four samples were corticosterone (range 37.25-51.26 ng ml^{-1}) and cortisol (range 32.57-52.24 ng ml^{-1}), followed by 17-OH-pregnenolone, dihydrotestosterone and pregnenolone. Stallion displayed the highest total steroids level (155.12 ng ml^{-1}), followed by gelding (133.68 ng ml^{-1}) and mare. Stallion serum displayed higher levels of 17-OH-pregnenolone and testosterone (27.24 and 4.80 ng ml^{-1} respectively) with respect to gelding (7.81 ng ml^{-1} - n.d.), but comparable amount of others steroids such as androstenedione, androsterone and pregnenolone. Level of testosterone in geldings is lower that LOQ (i.e. 50 pg ml^{-1}), and thus lower than the threshold regulated in plasma (100 pg ml^{-1} of free testosterone).[3] On the other side, the level found in stallion equine sample is quite high (4800 pg ml^{-1}) but estosterone is still not regulated in stallion. Stallion and gelding showed higher level of cortisol, corticosterone and 17-OH-pregnenolone with respect to mare, but comparable levels of pregnenolone. Only in the two mare serum samples we were able to detect estradiol (1.38-5.74 ng ml^{-1}) at conspicuous levels.

Conclusions

In this paper an ultra-high-performance liquid chromatography– tandem mass spectrometry (UHPLC–MS/MS) method has been developed, permitting the detection of 17 steroids in equine serum samples. The procedure is fast and intuitive, the sample preparation is easy, with deproteinization inside the vials followed by centrifugation and instrumental analysis. The present analytical method exhibited good performances in terms of specificity, sensitivity (LOQ in the range 0.0055-5.5 ng ml^{-1}) and linearity. Another advantage of this developed analytical protocol is the simultaneous monitoring of a very large number of different

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hormones presenting various steroid substructures in a short time (11 minutes chromatographic run). Most of the compounds chosen in the current method are often different from those reported in literature and not usually included in analytical methods. The method permitted to detect most of steroid esters in equine serum. After full method validation, the procedure has been successfully applied to the analysis of equine serum samples (mare, gelding and stallion). The main steroids found in the four samples were corticosterone and cortisol, followed by 17-OH-pregnenolone, dihydrotestosterone and pregnenolone. Aldosterone, DHEAS, DHEA and estrone were detected in any samples. In conclusion, the present method allows identification and quantification of steroids and it could be used when fraudulous use is suspected in racing animals or in equine trade.

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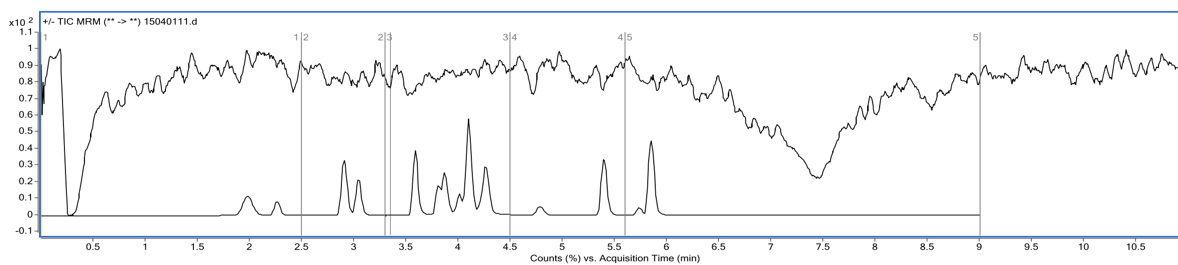

Figure 1. Postcolumn infusion of steroids serum free and a mixture standard of all searched compounds in HPLC-grade methanol.

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Table 1. UHPLC-MS/MS acquisition parameters (MRM mode) used for the analysis of steroidal hormones and metabolites.

Compound	Abbreviation	Time Widow (min)	Precursor Ion (*m*/z)	Product Ion[a] (*m*/z)	Fragmentor (V)	Collision Energy (V)	Dwell Time (sec)	Polarity
Cortisol	CORT	1.3-2.5	363.01	121.1	136	24	350	POS
				327.2	136	12		
Aldosterone	ALDO	1.3-2.5	361.41	343.2	116	16	350	POS
				315.2	116	20		
11-Deoxycortisol	11-DOC	2.5-3.3	347.51	109.1	141	32	200	POS
				97.2	141	28		
Corticosterone	CoCo	2.5-3.3	347.01	329.2	111	12	200	POS
				121.1	111	28		
Dehydroepiandrosterone Sulfate	DHEAS	2.5-3.3	366.99	97	165	32	200	NEG
				80	165	50		
17-OH-Progesterone	17-OH PROG	3.3-4.5	331.01	313.2	102	4	100	POS
				57.2	102	24		
11-DeoxyCorticosterone	11-DCC	3.3-4.5	331.01	97.1	117	20	100	POS
				109.1	117	32		
17-OH-Pregnenolone	17-OH PRE	3.3-4.5	297.1	105.1	150	36	100	POS
				91.2	150	48		
Dehydroepiandrosterone	DHEA	3.3-4.5	289.01	271.2	101	4	100	POS
				253.2	101	4		
Testosterone	TESTO	3.3-4.5	289.01	97.1	131	20	100	POS
				109.1	131	28		
Androstenedione	ANDD	3.3-4.5	287.01	97.1	131	24	100	POS
				109.1	131	24		
Estrone	ESTRO	3.3-4.5	271.01	253.1	92	8	100	POS
				157.1	92	20		
Estradiol	ESTRA	3.3-4.5	255.01	159.1	102	16	100	POS
				133.1	102	20		
Androsterone	ANDRO	4.5-5.6	291.41	273.2	78	4	350	POS
				255.2	78	12		
Dihydrotestosterone	DHT	4.5-5.6	273.1	255.3	159	15	350	POS
				147.0	159	16		
Progesterone	PRO	5.6-7.0	315.01	97.1	126	24	400	POS
				109.1	126	24		
Pregnenolone	PRE	5.6-7.0	299.01	281.2	111	8	400	POS
				105.0	111	40		
d_7-Aldosterone	d_7 - ALDO	1.3-2.5	368.3	350	135	15	350	POS
d_4-Cortisol	d_4 – CORT	1.3-2.5	367.01	121	135	25	350	POS
d_3-Testosterone	d_3 – TESTO	3.3-4.5	292	97	135	25	100	POS

[a]For each compounds, the product ions in the first row were used for the quantification, those in the second row were used for qualification.

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Table 2. Values of the concentrations used for method validation for each analyte.

Compound	LOD[a] ng ml-1	LOQ ng ml-1	C1 ng ml-1	CM ng ml-1	C2 ng ml-1	CU ng ml-1	U1 ng ml-1	U2 ng ml-1	U3 ng ml-1	U4 ng ml-1
CORT	0.002	0.0055	2.7	27.5	55	40	220	330	440	550
ALDO	0.03	0.055	0.275	2.75	5.5	11	22	33	44	55
11-DOC	0.2	0.55	2.75	27.5	55	110	220	330	440	550
CoCo	0.02	0.05	0.25	2.5	5	10	20	30	40	50
DHEAS	0.2	0.55	2.75	27.5	55	110	220	330	440	550
17-OH PROG	0.2	0.55	2.75	5.5	11	22	44	66	88	110
11-DCC	0.2	0.55	2.75	27.5	55	110	220	330	440	550
17-OH PRE	0.3	1.1	2.2	22	44	88	176	264	352	440
DHEA	0.5	1.1	5.5	55	110	220	440	660	880	1100
TESTO	0.02	0.05	0.275	2.75	5.5	11	22	33	44	55
ANDD	0.025	0.05	0.25	2.5	5	10	20	30	40	50
ESTRO	0.05	0.11	0.55	5.5	11	22	44	66	88	110
ESTRA	0.02	0.055	0.275	2.75	5.5	11	22	33	44	55
ANDRO	0.05	0.11	0.55	5.5	11	22	44	66	88	110
DHT	0.02	0.055	0.275	2.75	5.5	11	22	33	44	55
PROG	0.2	0.55	2.75	27.5	55	110	220	330	440	550
PRE	2	5.5	11	110	220	440	880	1320	1760	2200

[a]LOD values were calculated and reported also if they were not used for validation measurement

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Table 3. Intra- and interday precision expressed in CV% (Coefficient of Variation)

Compound	LOQ		CM		CU		U3	
	Intraday (CV%)	Interday (CV%)	Intraday (CV%)	Interday (CV%)	Intraday (CV%)	Interday (CV%)	Intraday (CV%)	Interday (CV%)
CORT	10.62	13.46	6.31	7.39	2.46	3.95	0.86	1.32
ALDO	16.23	17.69	9.03	10.14	6.30	8.04	3.39	4.32
11-DOC	14.21	16.74	6.51	8.20	6.60	7.44	5.89	6.66
CoCo	15.60	16.64	9.60	10.70	2.61	3.52	1.02	2.21
DHEAS	15.69	16.67	14.50	17.60	6.14	18.15	3.09	7.93
17-OH PROG	16.86	17.08	6.20	7.53	6.74	7.34	5.30	6.54
11-DCC	10.10	13.86	7.52	10.42	1.40	4.34	0.33	2.12
17-OH PRE	13.01	14.24	5.31	8.91	2.98	5.06	1.02	4.03
DHEA	15.59	17.10	11.35	16.42	3.54	17.80	1.87	6.78
TESTO	17.11	17.82	6.68	14.52	5.31	7.94	2.10	5.65
ANDD	17.19	17.57	9.30	18.74	7.69	9.45	4.32	6.22
ESTRO	16.36	17.28	15.51	18.53	11.99	18.02	7.02	9.21
ESTRA	11.52	17.38	10.91	16.65	6.16	14.83	4.22	5.54
ANDRO	9.84	15.90	13.52	18.21	8.36	9.68	6.11	7.87
DHT	13.97	17.05	9.52	18.39	4.49	8.29	2.02	3.09
PRO	16.57	16.99	4.92	8.03	4.13	4.62	2.76	3.04
PRE	13.73	16.33	8.61	8.99	6.13	6.38	4.12	5.12

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Table 4. Intra- and interday accuracy expressed in RE% (Relative Error)

Compound	C1		C2		U3	
	Intraday (RE%)	Interday (RE%)	Intraday (RE%)	Interday (RE%)	Intraday (RE%)	Interday (RE%)
CORT	6.73	6.94	1.92	2.41	1.76	1.93
ALDO	10.90	11.46	4.44	4.81	2.34	3.83
11-DOC	9.42	9.77	3.55	4.72	2.55	2.73
CoCo	7.63	10.21	6.46	7.25	3.41	4.24
DHEAS	8.71	9.52	3.27	5.58	1.27	3.27
17-OH PROG	10.92	12.03	4.38	5.77	2.39	3.79
11-DCC	5.92	6.52	1.99	2.26	0.92	2.20
17-OH PRE	12.95	13.72	6.03	6.14	4.04	4.12
DHEA	8.46	9.72	6.24	6.53	3.25	3.92
TESTO	13.24	13.43	4.45	5.02	2.11	3.11
ANDD	11.23	10.85	6.36	7.72	4.37	4.94
ESTRO	12.62	13.90	5.37	7.31	3.38	3.65
ESTRA	9.11	13.70	5.71	6.60	3.67	4.66
ANDRO	8.41	9.48	5.21	5.62	4.16	4.67
DHT	10.70	11.17	3.12	4.66	2.26	3.68
PRO	9.52	10.72	4.03	4.34	2.02	2.85
PRE	7.05	9.01	3.82	5.29	2.22	3.24

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Table 5. Percent recovery and reproducibility at two fortification levels.

Compounds	CM		C1	
	Recovery *n*=3	CV (%)	Recovery *n*=3	CV (%)
CORT	96.64	1.36	91.03	4.22
ALDO	95.09	0.94	89.64	5.80
11-DOC	97.59	1.53	92.06	5.61
CoCo	92.79	5.04	87.42	5.79
DHEAS	92.51	1.96	95.79	3.72
17-OH PROG	97.66	0.91	92.10	6.02
11-DCC	95.85	3.10	90.16	2.93
17-OH PRE	91.84	2.05	86.58	5.23
DHEA	96.03	3.08	90.56	6.44
TESTO	95.69	0.78	91.99	6.07
ANDD	95.70	1.06	99.39	6.16
ESTRO	92.02	2.69	86.66	3.43
ESTRA	93.53	1.01	88.09	5.24
ANDRO	95.71	3.59	89.98	8.37
DHT	91.05	1.08	85.60	5.72
PRO	95.27	2.57	89.78	7.36
PRE	93.09	0.65	87.74	4.44

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Table 6. Content of steroidal hormones in equine serum samples[a,b], expressed in ng ml^{-1}

Analytes	Mare	Mare	Stallion	Gelding
CORT	32.57	36.14	50.76	52.24
ALDO	n.d.	n.d.	n.d.	n.d.
11-DOC	n.d.	0.25	0.25	0.08
CoCo	42.38	37.25	51.13	51.26
DHEAS	n.d.	n.d.	n.d.	n.d.
17-OH PROG	0.04	n.d.	0.11	0.08
11-DCC	0.03	n.d.	n.d.	0.03
17-OH PRE	n.d.	5.32	27.24	7.81
DHEA	n.d.	n.d.	n.d.	n.d.
TESTO	0.04	0.04	4.80	n.d.
ANDD	n.d.	0.18	0.30	0.17
ESTRO	n.d.	n.d.	n.d.	n.d.
ESTRA	1.38	5.74	n.d.	n.d.
ANDRO	0.26	4.05	2.02	0.91
DHT	n.d.	12.62	9.62	10.92
PRO	0.42	3.11	0.11	0.08
PRE	3.99	9.03	8.78	9.37
Total	81.10	113.72	155.12	133.68

[a]Each sample was analyzed in triplicate. Percent RSDs in all cases were lower than 13.66%.
[b]nd: not detectable.

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