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Development of a Multi-class Steroid Hormone Screening Method using Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS)

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Abstract

Monitoring complex endocrine pathways is often limited by indirect measurement or measurement of a single hormone class per analysis. There is a burgeoning need to develop specific direct-detection methods capable of providing simultaneous measurement of biologically relevant concentrations of multiple classes of hormones (estrogens, androgens, progestogens, and corticosteroids). The objectives of this study were to develop a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for multi-class steroid hormone detection using biologically relevant concentrations, then test limits of detection (LOD) in a high-background matrix by spiking charcoal-stripped fetal bovine serum (FBS) extract. Accuracy was tested with National Institute of Standards and Technology Standard Reference Materials (SRMs) with certified concentrations of cortisol, testosterone, and progesterone. 11-Deoxycorticosterone, 11-deoxycortisol, 17-hydroxypregnenolone, 17-hydroxyprogesterone, adrenosterone, androstenedione, cortisol, corticosterone, dehydroepiandrosterone, dihydrotestosterone, estradiol, estriol, estrone, equilin, pregnenolone, progesterone, and testosterone were also measured using isotopic dilution. Dansyl chloride (DC) derivatization was investigated maintaining the same method to improve and expedite estrogen analysis. Biologically relevant LODs were determined for 15 hormones. DC derivatization improved estrogen response two- to eight-fold, and improved chromatographic separation. All measurements had an accuracy 14 % difference from certified values (not accounting for uncertainty) and relative standard deviation 14 %. This method chromatographically separated and quantified biologically relevant concentrations of four hormone classes using highly specific fragmentation patterns and measured certified values of hormones that were previously split into three separate chromatographic methods.

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Conflict of Interest
The authors declare that they have no conflict of interest.

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Keywords

Steroid hormones; Liquid chromatography tandem mass spectrometry; Estrogens; Androgens; Progestogens; Corticosteroids

Introduction

The endocrine system controls signaling pathways that direct critical physiological processes such as development, reproduction, and metabolism [1]. A major group of signaling molecules of the endocrine system is the steroid hormone family. Steroid hormones are defined as endogenous hormones synthesized from a cholesterol backbone (a planar tetracyclic ring structure with rings usually denoted as A through D) and are enzymatically transformed into different classes of steroids (Figure 1) [1, 2]. Subtle changes in steroid structure (usually in number and location of carbonyl and hydroxyl functional groups) lead to drastic differences in physiological function [3]. There are four classes of steroid hormones, which include estrogens, androgens, progestogens, and corticosteroids. These classes are often grouped according to both structure and by which genomic receptors they bind to induce biological function [4]. Estrogens (18 carbons with an aromatic A ring) are typically denoted as female reproductive hormones, androgens (19 carbons) as male reproductive hormones, progestogens (21 carbons) as pregnancy hormones, and corticosteroids (21 carbons) as stress hormones [1]. Despite their categorization, steroid hormones have multiple functions that cross the general physiological categories, and are synthesized through a cascade-like pathway (meaning one hormone is required to produce the next) [4]. For example, while estrogens are thought of as "female hormones" and androgens thought of as "male hormones," [3] almost all vertebrate males and females have detectable levels of both androgens and estrogens. Further, estrogens cannot be produced without first producing androgens. Cortisol, a corticosteroid typically associated with stress, has a reproductive function as it is associated with the initiation of labor and birth, whereas progestogens are often monitored for adrenal dysfunction. Because steroids have multiple physiological functions and a cascade-like synthesis pathway, studying hormone milieus would greatly enhance the knowledge of which steroid hormones are involved in key physiological functions. Additionally, most steroid hormones are present in all vertebrates, but different species regulate them at different concentrations. For example, dihydroprogesterone is the primary progestogen for pregnancy in elephants, whereas progesterone is the primary pregnancy hormone in humans [5]. By measuring only progesterone, the reproductive physiology of the elephant cannot be fully understood. However, a comprehensive steroid hormone profile could be used to understand the varying physiologies of species from fish to humans.

The ability to monitor multiple alterations in hormone pathways is currently limited by the scope and scale of the traditional measurement methods typically used in biological studies. Radioimmunoassays (RIAs) and enzyme-linked immunosorbant assays (ELISAs) are the most commonly employed approaches for hormone measurement. These assays are generally extremely sensitive (capable of low pg/mL detections) due to the indirect, immunoglobulin-based measurement technologies that bind the hormone and amplify that

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signal through an enzymatic change or radioactive signal. However, the amplification technologies that give the immunoassays such sensitivity are often less reliable than direct quantification methods. Antibody cross-reactivity, matrix interferences, reliance on external calibration, and poor inter method reproducibility are all common issues due to the indirect detection methods of immunoassays [6–8]. Further, RIAs and ELISAs are designed for the explicit measurement of a single hormone per assay, thus limiting the ability to measure multiple hormones per sample.

While gas chromatography (GC) offers excellent chromatographic resolution and the benefit of multi-class profiling potential, the analysis of steroids using this method has its pitfalls. Many steroids have to be derivatized for GC-MS (mass spectrometry) which usually entails derivatizing the hydroxyl groups [9]. Reproducibility becomes an issue due to incomplete derivatization of compounds such as cortisol that have three hydroxyl groups. Therefore, the adoption of liquid chromatography (LC) separation using the underivatized compound remains more advantageous to GC-based methods.

Due to advances made in LC coupled with tandem mass spectrometry (MS/MS), LC-MS/MS methods are now preferred for the direct measurement of steroid hormones; however, this technique has its difficulties and many purported multi-class methods have limitations. Steroids have very similar structures and fragmentation patterns, making the separation of multiple steroids, some of which have identical masses, difficult yet critical. Additionally, many of the hormones that have been measured by LC-MS/MS did so by monitoring the precursor ion minus 18 amu [10], usually indicating a loss of water. The loss of water is a common fragmentation pattern not specific to hormones, which could lead to misidentifications in complex biological matrices where background ion detection may impair measurement. Therefore, clear separation and unique fragmentation patterns are necessary for accurate steroid hormone measurement.

Most studies feature methods aimed at monitoring single hormones or specific hormone classes (e.g. estrogens or androgens) within a given sample analysis [11–17]. Estrogens present an additional complication for multi-class methods in that endogenous circulating concentrations in serum are typically in the low pg/mL range. In addition, estrogens are relatively polar in comparison to many of the other steroid classes making fragmentation of these compounds in electrospray suboptimal. Therefore, estrogens are often not included in the existing multi-class LC-MS/MS methods for serum. To our knowledge, few publications describe a method using LC-MS/MS detection of all four classes in serum [18, 10, 19] and those that claim this ability lack sensitivity for biologically relevant concentrations of multiple estrogens or contains proprietary information which requires the purchase of a commercial kit to conduct the analysis. There exists a need within the hormone measurement community for an improved measurement method for the multi-class detection of steroid hormones capable of highly sensitive and specific measurements in biological matrices using LC-MS/MS.

This manuscript outlines a method that allows for multi-class analysis using a single extraction and chromatographic method with high specificity for steroid hormones and hormone-like structures validated through the use of standard reference materials (SRMs)

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from the National Institute of Standards and Technology (NIST). The aims of this research were four-fold: 1) develop an LC-MS/MS method to measure biologically relevant ranges of multiple steroid hormone standards (from four steroid classes previously mentioned) in a single method using only unique fragmentation patterns (i.e. not monitoring the loss of water); 2) test the feasibility of this approach in detecting various concentrations of steroid hormones in a high-background hormone-stripped, fetal bovine serum (FBS) extract; 3) determine the enhancement of estrogen detection in both matrices through dansyl chloride (DC) derivatization without the need to change chromatographic parameters; 4) compare values derived from this method to certified concentrations of hormones from serum and plasma SRMs.

Materials and Methods

Materials

For the purpose of method development, a focus was placed on examining steroid hormones either with known biological action or present at critical points in steroid synthesis pathways (Figure 1). Neat sources of adrenosterone (AT), androstenedione (AE), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), testosterone (T), estradiol (E_2), estrone (E_1), estriol (E_3), equilin (EQ), progesterone (P_4), pregnenolone (P_5), 17-hydroxyprogesterone (17-OHP_4), 17-hydroxypregnenolone (17-OHP_5), cortisol (F), corticosterone (B), 11-deoxycorticosterone (DOC), and 11-deoxycortisol (S) were obtained for this study (manufacturer information in Online Resource 1). The structural diversity of these compounds includes 18 to 21 carbon structures, aromatic and non-aromatic A rings, A or B ring double bonds, zero to three carybonyl groups, and zero to three hydroxyl groups. EQ is unique to the classes as it is an equine estrogen that is widely used as a pharmaceutical during estrogen replacement therapy for postmenopausal women [20]. While not physiologically relevant to humans, EQ was incorporated to further explore the flexibility in analyzing non-human steroid-like structures. EQ is an excellent surrogate because it is not synthesized through the cholesterol pathway [21], is structurally different from most human steroids (18 carbons with an aromatic A ring and an additional double bond in the B ring), and informs the future use of this technique in the investigation of both wildlife endocrinology and pharmaceutical detection.

Internal standards (IS) representative of the major hormones, testosterone-$^{13}C_3$ (T-$^{13}C_3$), estradiol-$^{13}C_3$ (E_2-$^{13}C_3$), progesterone-$^{13}C_2$ (P_4-$^{13}C_2$), pregnenolone-$^{13}C_2d_2$ (P_5-$^{13}C_2d_2$), 17-hydroxyprogesterone-$^{13}C_3$ (17-OHP_4-$^{13}C_3$), and cortisol-d_4 (F-d_4), were used to assist in quantification of the hormones (see manufacturer details in Online Resource 1). A single mixture of all IS (300 ng/mL of each) was prepared. From this mixture, 250 μL was added gravimetrically to samples and calibrants yielding 75 ng of each IS. A new carbon labeled P_4 (a $^{13}C_3$ labeled IS as opposed to the $^{13}C_2$ used in the previous sections) became available during these analyses and was adopted for the SRM measurement portion of this study.

LC-MS/MS Method Optimization

All steroids were optimized on an AB Sciex API4000 QTRAP (Framingham, MA) hybrid triple quadrupole/linear ion trap mass spectrometer equipped with electrospray ionization

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(ESI, positive mode) in tuning mode. Individual steroids were directly injected and fragmentation patterns were evaluated at varying collision energies until two to four candidate product ions were identified and one product ion selected based on both compound and source specific parameter optimization (Table 1). A fragmentation pattern of a loss of 18 or 36 mass units was excluded as these were most likely a loss of water molecules. Instrument parameters were compared across all optimized fragmentation patterns and selected based on no major loss in detection for any compound analyzed (curtain gas = 207 kPa, temperature = 700° C, ion source gas 1 = 310 kPa, ion source gas 2 = 414 kPa, interface heater = On, collision gas = medium, and ion spray voltage = 5500 V).

A novel separation method was developed using a Restek Ultra Biphenyl column (250 mm × 4.6 mm, 5.0 μm particle size) heated at 35 °C on an Agilent 1200 Series HPLC system equipped with a binary pump and autosampler was used to separate steroid hormones. Sample injection volume was 5 μL and flow was 300 μL/min. Total LC analysis time was 40 min with a solvent gradient consisting of MeOH and acetonitrile (ACN), both containing 0.1 % formic acid. The gradient was as follows: MeOH 80 % to 55 % for 30 min, 55 % to 20 % for 1 min and held for 4 min, up to 80 % in 0.1 min, and held at 80 % for the remaining 9.9 min. Retention times for each compound were determined using multiple-reaction monitoring (MRM) (Figure 1). After optimization, measurements of all steroids and IS were monitored using scheduled multiple-reaction monitoring (sMRM).

Calibration and Limit of Detection

Initial quantitation experiments were conducted using standards of 17 endogenous steroid hormones prepared in MeOH at ten calibration levels in borosilicate glass culture tubes (n = 3 for each level). The 17 steroids were combined and used to prepare a ten point calibration curve ranging from 6 pg to 500 ng in methanol. Approximately 250 μL of IS mixture was gravimetrically added to each sample. These calibration standards were evaporated under a gentle stream of high-purity nitrogen and reconstituted in 200 μL of MeOH prior to analysis by LC-MS/MS. Sample analysis position was randomized along with two types of blanks, solvent blanks containing only MeOH and IS blanks containing only internal standards in MeOH. IS blanks were analyzed to obtain limits of detection (LOD) in the absence of matrix.

LODs were also determined in a charcoal stripped FBS in order to simulate an actual blood sample. Briefly, 2 mL of activated-charcoal stripped FBS (Invitrogen) was combined with 8 mL of cold MeOH to precipitate proteins. Aliquots were vortexed for 1 min and allowed to settle for 3 min before being frozen at −80 °C for 3 min. The supernatant was then pooled. FBS extract equivalent to 1 mL of whole FBS was then aliquoted into culture tubes and amended with calibration standards and IS as above (n = 5 for each level). Samples were then evaporated to dryness under nitrogen, reconstituted in 200 μL of MeOH, and transferred to autosampler vial inserts. FBS blanks were made by adding FBS extract and 500 μL of MeOH to replace the calibration standard and IS solutions. FBS IS blanks contained FBS extract, 75 ng of IS and 250 μL of MeOH to replace the calibration standard.

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Dansyl Chloride Derivatization

To aid in the detection of estrogens, a derivatization method (dansylation) was examined and the results were compared to those obtained from the underivatized estrogens. DC dertivatization was conducted for estrogens amended to methanol (three samples per level) and FBS (five samples per level).

The DC protocol was conducted according to Nelson et al. [6]. The dansylated sample was evaporated under nitrogen at 40 °C, reconstituted in 200 μL MeOH, vortexed for 10 s and transferred (avoiding any undissolved salts) to a 250 μL glass autosampler vial insert. Optimization of MS/MS fragmentation patterns and conditions were conducted for all DC-derivatized compounds using the same instrument parameters and chromatographic conditions the underivatized compounds.

Standard Reference Material (SRM) Measurement

This analysis was conducted on SRMs 971M and 971F, Hormones in Human Serum (male and female), and SRM 1950, Metabolites in Human Plasma. SRMs 971 and 1950 are certified for F, T, and P_4 and provide analysis of both a serum and plasma matrix. All SRMs were measured for AT, AE, DHEA, DHT, T, E_2, E_1, E_3, P_4, P_5, 17-OHP_4, 17-OHP_5, F, B, DOC, and S as well.

Sample extraction was performed using a modified solid phase extraction (SPE) outlined by Tai et al. and Budzinksi et al.[22, 13, 23] An internal standard (IS) mixture in methanol (MeOH) was added volumetrically (500 μL) to borosilicate glass culture tubes using airtight gas syringes and tracked gravimetrically. The IS mixture was then reduced to dryness under nitrogen before the addition of matrix to prevent precipitation of proteins in the serum and plasma. Serum (2 mL; n = 5), plasma (2 mL; n = 5), calibration standards, or MilliQ water for blanks were gravimetrically added into borosilicate culture tubes. Blanks were analyzed in triplicate and contained only the IS mixture that was reconstituted with MilliQ water. Sodium acetate buffer (4 mL, 0.01 M, pH 5) was added to the tubes, lightly vortexed, and then allowed to incubate at room temperature for 1 h. Supelclean LC-18 SPE cartridges (1 g × 6 mL) (Sigma, Bellefonte, PA) were used for extraction. Columns were conditioned with 5 mL methanol (MeOH), followed by 5 mL MilliQ water, and 1 mL acetate buffer (0.01 M, pH 5). Sample was then loaded after the incubation period and washed with 12 mL of MilliQ water followed by 5 mL of a solution of 80:20 MilliQ water and acetonitrile. The sample was then eluted into a clean borosilicate culture tube with 2.5 mL of MeOH. The eluate was then evaporated to dryness on nitrogen at 40 °C, reconstituted in 200 μL of MeOH, vortexed for 10 s and transferred to a 250 μL glass autosampler vial insert in an amber autosampler vial.

For the analysis of estrogens, a dansyl chloride (DC) derivatization was conducted after LC-MS/MS measurement of the other steroid classes. An aliquot of 100 μL of extract was removed from the autosampler and added to a borosilicate culture tube for reaction. Derivatized samples were then reconstituted in 100 μL of methanol, vortexed for 10 s and transferred to a 250 μL glass autosampler vial insert in an amber autosampler vial.

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Quantitation

Integrations were conducted using Analyst v1.5.2 (AB Sciex). Data analyses were conducted using appropriate IS adjusted peak areas and masses (see Table 2 for steroid/IS pairing). A linear regression (y = mx + b) was performed on the range of the calibration standards that not only gave linearity (R^2 0.9996) but also represented the biologically significant reference range of the steroid in humans [24, 25].

The LODs were calculated using two approaches, statistical and empirical [26]. First, the sample noise was selected from the IS blanks containing only internal standard. Next, a linear regression was generated from the calibration curve and a statistical LOD (*LODst*) was extrapolated from the equation using:

$$LODst=\frac{(\text{mean noise area}+(3*\text{standard deviation of the noise})-b)\times ISm}{m}$$

Where b = the intercept, m = the slope, and ISm = the appropriate internal standard mass. The LOD_{st} was selected if the model's R^2 0.9998. Secondly, the empirical LOD (LOD_{em}) was based on the lowest calibration standard successfully measured which was greater than three times the standard deviation of the noise plus the mean noise area.

Response factors were calculated to determine the efficacy of the derivatization on the detection of estrogens using DC in comparison to the detection with no derivatization. T-$^{13}C_3$ internal standard was used as a surrogate internal standard because unlike the estrogen internal standards, the T-$^{13}C_3$ did not derivatize and had a constant response between the two analyses. Linear regressions were determined for the ratio of peak area of the estrogen and peak area of T-$^{13}C_3$ by the ratio of the mass of the estrogen by the mass of T-$^{13}C_3$ in the sample. The slope of this linear regression was defined as the response factor.

After screening of the SRMs, three calibration mixtures were produced containing E_2, E_3, E_1, S, B, 17-OH-P_4, T, DOC, AE, AT, and P_4. Because F concentrations were an order of magnitude greater than the other steroids analyzed, a separate calibration curve was constructed containing only F. For accuracy, T was measured on two ends of the calibration curves as the concentrations for 971F were in the pg/mL range while the other materials were in the ng/mL range. For increased accuracy, all calibration solutions were spiked with the IS mixture and treated identically to samples during the extraction process. Masses of each analyte were calculated using the linear or quadratic regression of at least a three point calibration curve that bracketed the sample peak area ratios (area of the analyte over area of the appropriate internal standard). Concentrations were determined by dividing the calculated mass of each analyte by the extracted sample mass. Again, the LOD was determined as the lowest calibration standard or three times the standard deviation plus the mean of the internal standard blanks for each analyte, and the highest value was denoted as the limit of quantitation (LOQ) [26]. Percent differences were calculated using the mean measured value and the certified values which do not take in to account the range of uncertainty; however, given the small ranges of uncertainty on the certified values, percent differences were included for comparison.

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Results and Discussion

Chromatographic separation of 17 endogenous hormones and seven internal standards was achieved a using unique solvent and stationary phase combination (Figure 2). Most publications of steroid hormone analysis focus on certain hormones or classes of hormones chromatographically separated with a methanol:water solvent phase and use C18 columns [17]. We found that using a biphenyl column with a methanol:acetonitrile mobile phase yielded better separation by capitalizing on the difference in affinities for acetonitrile by the number of carbonyls versus hydroxyl groups of the highly structurally similar steroids. Many steroids including T and DHEA (289 amu), 17-OH-P_4 and DOC (331 amu), P_4 and 17-OH-P_5 (315 amu), and B and S (347 amu) have the same precursor ion mass and generate similar fragments (121 and 97 are common steroid fragments). These compounds were clearly separated by elution time, thereby reducing errors in quantification. The use of these solvents instead of water helped maintain excellent peak resolution, and potentially reduces laboratory variability generated by the source of water.

Calibration curves with concentrations over physiologically relevant ranges were constructed successfully for all but two hormones (Online Resource 2). DHEA and DHT were not detectable within biological ranges using this methodology; therefore, LODs could not be calculated (Table 2). DHEA is a biologically important androgen; however, measurements of DHEA-sulfate (DHEA-S), which circulates at higher concentrations in the blood, is more stable than DHEA in the blood and is often used as a proxy for DHEA [27]. Chromatography and MS/MS fragmentation would have to be explored further to add DHEA-S to this method. DHT is a highly important androgen for development, but most of the enzymatic activity to produce DHT from T occurs within target tissues, such as the skin and prostate [28]. Because DHT is synthesized and acts locally, serum DHT measurements may not be an informative measurement, and could be excluded from this method in the future.

Detection of most of the selected steroids did not appear to be compromised by the use of an FBS matrix (Table 2). Among the underivatized compounds, B was the only steroid that could be quantified only in MeOH and not in FBS. This was because the *LOD* for B in FBS was above all except the highest calibration standard (500 ng/mL) possibly indicating residual B in FBS. Additionally, the slope for FBS detection was greatly increased in FBS (57.625 in methanol to 106.86 in FBS; Supplementary Tables 2 and 3) indicating a potential matrix interference or more likely residual DOC in the bovine serum as this is the precursor hormone to the production of B. However, the *LOD* for B in MeOH was considerably lower (0.16 ng/mL). In humans, B is found in low concentrations (approximately 7 ng/mL), but B is a major hormone in many other species including bovines (approximately 30 μg/mL) [29, 30]. In non-human species, the calibration curve should be adjusted to higher concentrations, but given the performance of B and DOC detection in the higher ranges of the MeOH samples, this should not be an issue.

The developed method was able to circumvent the issues of low biological concentrations and poor ionization and fragmentation of estrogens by using a DC derivatization on the same extract under the same chromatographic and instrument conditions as the underivatized

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method, limiting the amount of labor to add these compounds to the analysis. DC derivatization performed well in enhancing detection of all measured estrogens. The response curves were greatly enhanced (two- to eight-fold) for estrogens with DC derivatization (Figure 3). Additional benefits to DC-derivatization were also exhibited, which included improved chromatography. Underivatized EQ and E_1 (a difference of two mass units) showed poor separation with current LC method; however, after DC derivatization, complete separation of EQ and E_1 was achieved (Figure. 2). Further, the LODs of E_3 and E_1 could not be calculated in the FBS matrix without DC derivatization. After derivatization, E_1 had an LOD of 200 pg/mL and E_3 an LOD of 400 pg/mL. An additional benefit to E_1 derivatization was that potential interferences in the measurement of underivatized E_1 in FBS (change in slope from 41.539 in methanol to 79.088 in FBS) were eliminated through derivatization (slopes equal 24.790 and 28.771 in methanol and FBS respectively; Supplemental Tables 2 and 3).

The purpose of using an extracted FBS was to examine the efficacy of the method in a high background non-human sample. Specialized extractions for steroid hormones, rather than a non-specific protein precipitation, would greatly enhance detection capabilities. Therefore, we continued with an analysis of human serum and plasma SRMs cleaned by SPE. LODs were greatly improved through the use of a simple SPE clean-up procedure compared to the FBS matrix (Table 2). Additionally, inclusion of calibration standards of lower concentrations than previously analyzed in the experiments lowered the LOD for some compounds (DOC, AE, E_2, E_3, E_1, 17-OH-P_4; see Online Resources 2 to 4 for calibration curve information). Analysis of F, T, and P_4 had good agreement (percent differences of less than 8.5 %, 13.3 %, and 2.85 % respectively) with SRM certified values for human male and female serum (SRMs 971M and 971F) as well as for human plasma (SRM 1950; Figure 4 and Table 3) indicating the accuracy of this single method compared to the three separate methods used to certify the SRMs.

Only three of the 13 final compounds analyzed in the SRMs were not reliably quantifiable (Table 3). These were DOC, S, and E_3, which were present, but were more variable RSD > 20%). Quantification of E_3 lacked precision most likely because of the low concentrations in the samples tested. Most of the estrogens remain in the low pg/mL ranges except leading up to ovulation (E_2) or during pregnancy (E_3) [31, 24]. Therefore, this method could be used to examine pregnancy in humans and wildlife when E_3 concentrations are elevated. Additionally, B quantification lacked precision for SRM 1950 and 971F, again most likely from being at the lower limits of quantification in these materials, because quantification was possible at higher concentrations in SRM 971M.

Estrogens were quantified in all three matrices. E_2 concentrations were too low to detect in the pooled male sample, SRM 971M, with this method. However, one note about the measurement of these SRMs is that these are pooled samples that have potentially incurred one freeze-thaw cycle in preparation. Degradation of some hormones that undergo freeze-thaw is expected, particularly E_2 and E_1 which have been shown to decrease in concentration with each freeze-thaw cycle [32]. Therefore, the concentrations reported here are lower than would be expected and E_2 concentrations in men may still be measurable in samples that have only been frozen once before analysis.

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A horse estrogen, EQ, used as a pharmaceutical, was separated and measured displaying the method's utility for additional analytes including steroid hormones found in other vertebrate species. Future analyses have the potential to include11-keto-testosterone from fish or synthetic steroids of similar structure such as ethinyl estradiol as demonstrated by the quantification of disparate but similar steroid structures. From compounds with three carbonyl (AT) to three hydroxyl (E_3) groups, this method demonstrated an enhanced capability in detecting a wide range of steroid hormones that has the potential to be used for human and wildlife endocrinology as well as pharmaceutical analysis.

This method allows for the accurate and precise measurement of T, P_4, and F in a single method. The measured hormones had RSDs under 14 % and a percent differences with certified values on both human male and female serum (SRM 971) matrix as well as a human plasma (SRM 1950) matrix of less than 12 %. Additionally, it provided precise measurement of E_2, E_1, 17-OH-P_4, AE, and AT, but measurements of S and B are more variable and dependent on concentrations of the hormones in the material. The method did not measure E_3 or DOC concentrations well, presumably due to low concentrations in the materials. Further testing of these hormones in other samples such as pregnant (E_3) or stressed (DOC, S, and B) individuals may increase concentrations of these hormones to quantifiable concentrations.

This method was flexible in its ability to detect multiple classes of steroid hormones with relatively minimal clean-up and is a promising method for the measurement of biologically relevant concentrations of hormones. We have shown this method to be effective in the direct measurement of several steroids from different classes, including the difficult to quantify estrogens, using a single methodology. Additionally, the method successfully quantified certified concentrations of T, F, and P_4 in three SRMs (male serum, female serum, and plasma matrices) using only one method as opposed to the previously required three separate methods. The chromatographic separation using the MeOH:ACN gradient on a biphenyl column, in combination with sMRM monitoring of steroid specific fragmentation patterns, allowed for separation and accuracy in peak measurement, which is essential for comprehensive steroid profiling. In the future we intend to explore a reduction in the amount of material needed for analysis. However, given the number of hormones measured in this method, the 2 mL currently required is not excessive. Additionally, this extraction method has the capability to be fully automated with SPE automation technology currently on the market, which would further reduce manpower and time to complete analysis.

The utility of this method is far reaching in its ability to measure endpoints from hormone pathways in a single sample as opposed to the one-at-a-time approach of immunoassays which becomes particularly useful when multiple samples are difficult or costly to collect and store. Using this method, a single frozen aliquot of serum or plasma yielded the same information as 11 to 12 individual immunoassays, saving time and labor. By measuring suites of hormones instead of a few carefully selected hormones, knowledge of complex biological events can be increased, which in turn aids in diagnosis of endocrine disruption of development, reproduction, and behavior.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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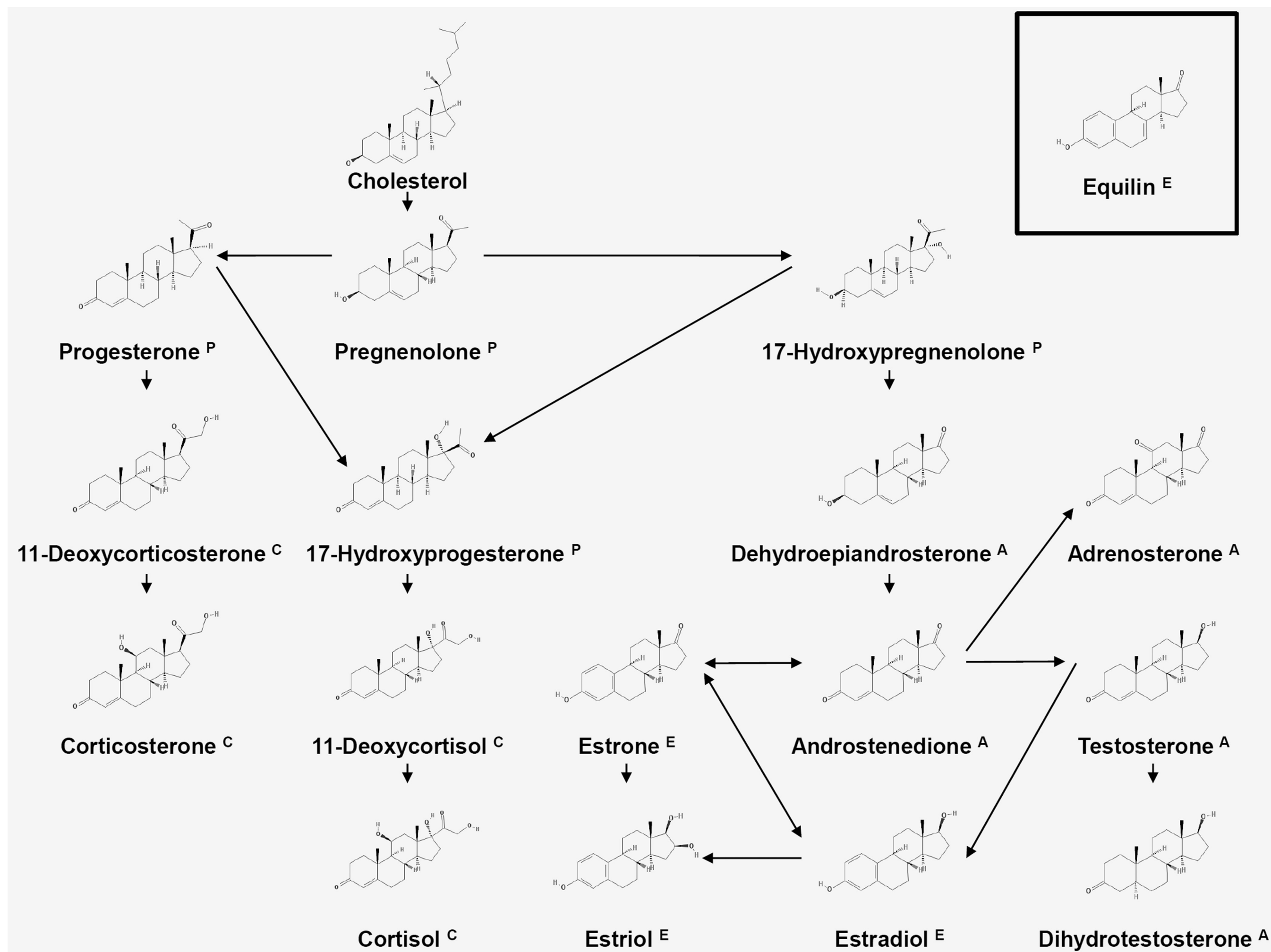

Fig. 1.

Steroid hormone structures and synthesis pathways. Arrows represent enzymatic reactions between precursor and product steroids. Superscript letters represent hormone classification. P = progestogen, C = corticosteroid, A = androgen, E = estrogen. Equilin has an alternate synthesis pathway and is therefore displayed structurally, but not included in this synthesis pathway. Chemical structures are from PubChem.

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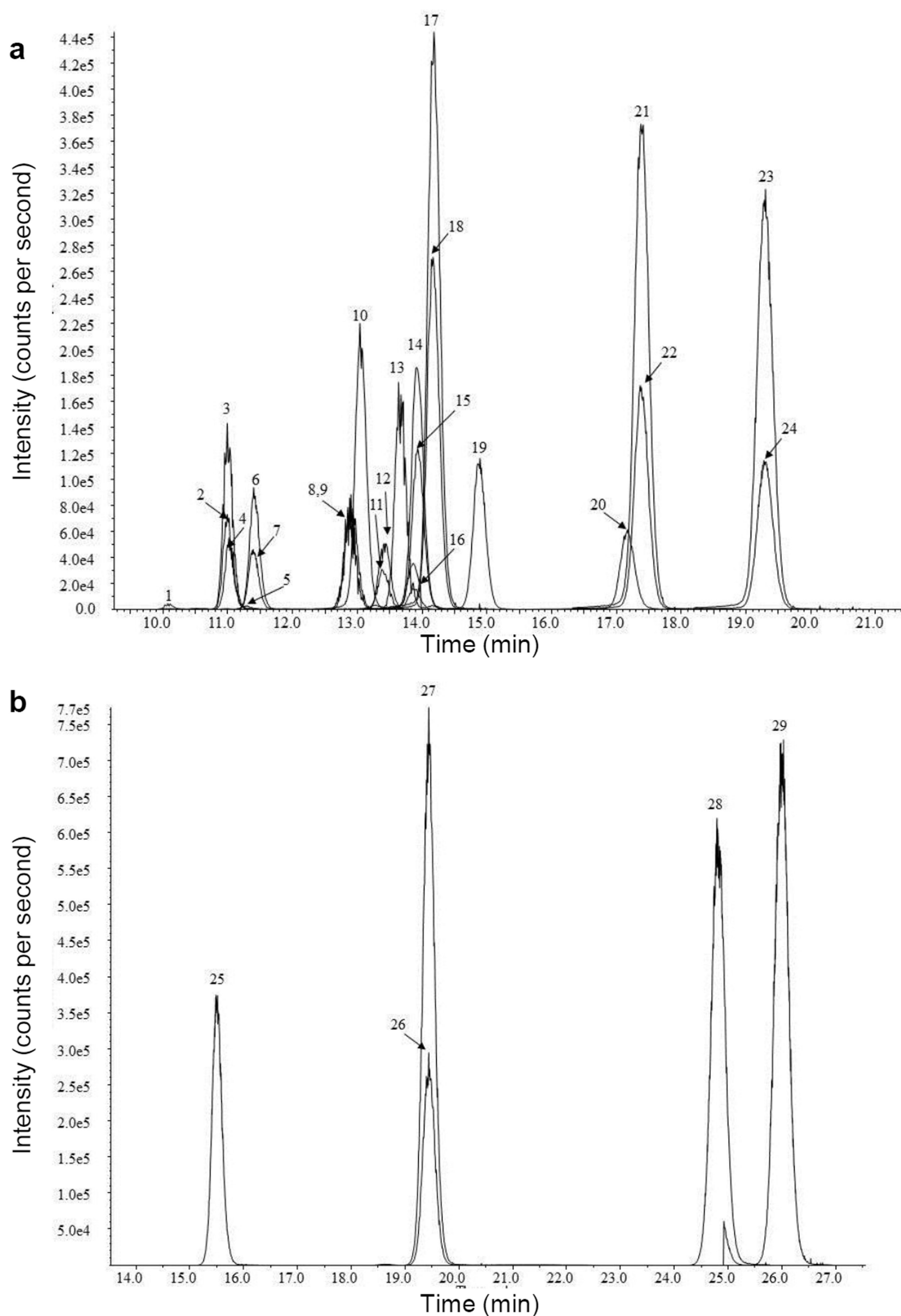

Fig. 2.

Chromatograms for separation of steroid hormones at 100 ng calibration standard concentration. (a) Underivatized. (b) Dansyl chloride derivatized. 1. Estriol (E_3), 2. Estradiol-$^{13}C_3$ (E_2-$^{13}C_3$), 3. Estradiol (E_2), 4. 17-OH-Pregnenolone (17-OH-P_5), 5. Dehydroepiandrosterone (DHEA), 6. Cortisol (F), 7. Cortisol- d_4 (F-d_4), 8. Equilin (EQ), 9. Estrone (E_1), 10. 11-Deoxycortisol (S), 11. Pregnenolone-$^{13}C_2d_2$ (P_5-$^{13}C_2d_2$), 12. Pregnenolone (P_5), 13. Dihydrotestosterone (DHT), 14. 17-OH-Progesterone (17-OH-P_4), 15. 17-OH-Progesterone-$^{13}C_3$ (17-OH-P_4-$^{13}C_3$), 16. Corticosterone (B), 17. Testosterone

(T), 18. Testosterone-$^{13}C_3$, (T-$^{13}C_3$), 19. Adrenosterone (AT), 20. 11-Deoxycorticosterone (DOC), 21. Androstenedione (AE), 22. Androstenedione-$^{13}C_3$ (AE-$^{13}C_3$), 23. Progesterone (P_4), 24. Progesterone-$^{13}C_2$ (P_4-$^{13}C_2$), 25. DC-Estriol (DC-E_3), 26. DC-Estradiol (DC-E_2), 27. DC-Estradiol-$^{13}C_3$,(DC-E_2-$^{13}C_3$), 28. DC-Equilin (DC-EQ), 29. DC-Estrone (DC-E_1).

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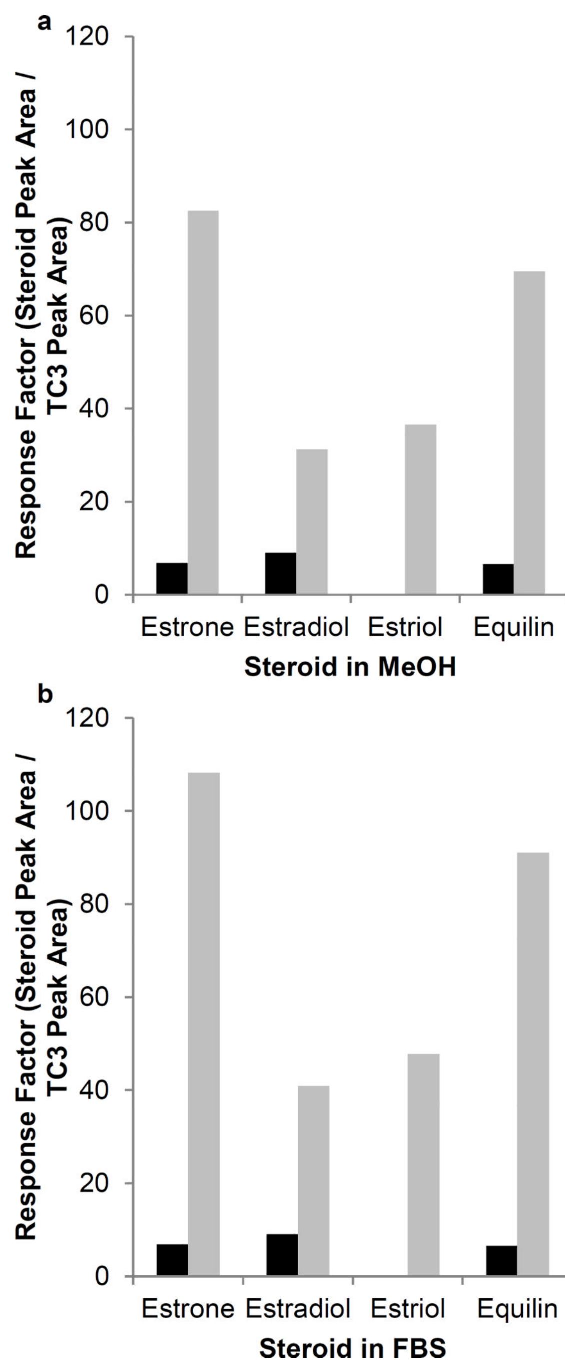

Fig. 3.

Dansyl chloride derivatization response factors. Underivatized estrogens are in black. Dansyl chloride derived estrogens are in grey. Response factors in methanol (a) and fetal bovine serum (b).

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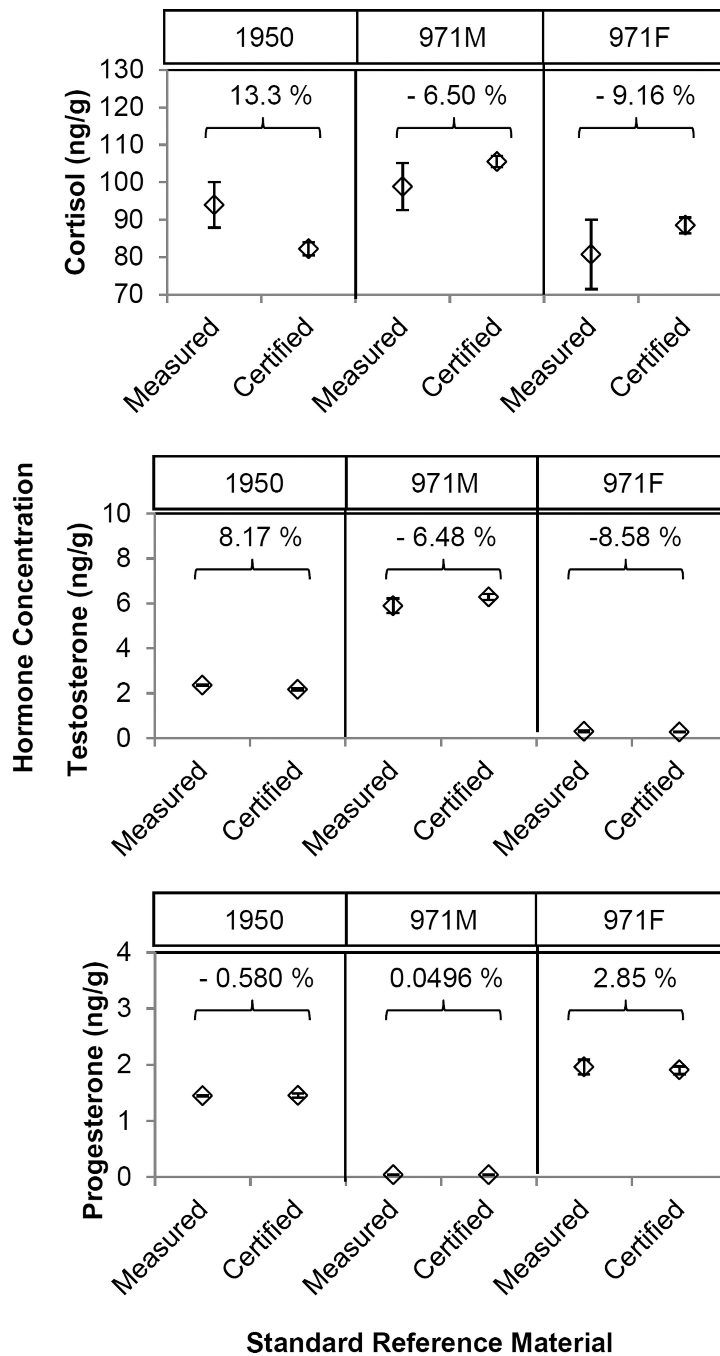

Fig. 4.

Measured and certified concentrations of cortisol, testosterone, and progesterone for three standard reference materials (SRMs), SRM 1950 (human plasma), SRM 971M (human male serum), and SRM 971F (human female serum). Error bars are one standard deviation for measured values and 95% expanded uncertainty for certified values. Numbers above brackets are the percent difference between the measured and certified values.

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Table 1

Parameters for steroid analysis using liquid chromatography tandem 522 mass spectrometry.

Steroid Common Name	Abbreviations	Absolute Mass (*m/z*)	Ion/Reaction	Precursor Ion (m/z)	Product Ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)	Retention Time (min)
Androgens										
Adrenosterone	AT	300.4	$[M+H]^+$	301.0	121.2	75	10	30	10	14.81
Androstenedione	AE	286.4	$[M+H]^+$	287.2	97.2	100	15	30	12	17.33
Androstenedione-$^{13}C_3$	AE-$^{13}C_3$	289.4	$[M+H]^+$	290.6	100.2	75	10	40	5	17.33
Dehydroepiandrosterone	DHEA	288.4	$[M+H-2H_2O]^+$	253.3	197.3	75	10	25	10	11.21
Dihydrotestosterone	DHT	290.4	$[M+H]^+$	291.3	255.4	75	10	25	10	13.62
Testosterone	T	288.4	$[M+H]^+$	289.1	97.2	100	15	30	12	14.11
Testosterone-$^{13}C_3$	T-$^{13}C_3$	291.6	$[M+H]^+$	292.6	112.2	75	10	40	5	14.08
Estrogens										
Equilin	EQ	268.4	$[M+H]^+$	269.2	211.2	75	10	25	10	12.83
Estradiol	E_2	272.4	$[M+H-H_2O]^+$	255.3	159.2	75	10	25	10	10.91
Estradiol-$^{13}C_3$	E_2-$^{13}C_3$	275.4	$[M+H-H_2O]^+$	258.2	162.2	50	10	35	10	10.91
Estriol	E_3	288.4	$[M+H-H_2O]^+$	271.3	133.4	75	10	25	10	10.01
Estrone	E_1	270.4	$[M+H]^+$	271.1	159.3	75	10	30	10	12.84
Progestogens										
17-Hydroxypregnenolone	17-OH-P_5	332.5	$[M+H-H_2O]^+$	315.4	297.3	40	5	20	5	10.97
17-Hydroxyprogesterone	17-OH-P_4	330.4	$[M+H]^+$	331.2	97.2	75	10	25	10	13.85
17-Hydroxyprogesterone-$^{13}C_3$	17-OH-P_4-$^{13}C_3$	333.4	$[M+H]^+$	334.6	100.1	40	5	40	5	13.88
Pregnenolone	P_5	316.5	$[M+H-H_2O]^+$	299.4	161.4	75	15	25	10	13.37

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Steroid Common Name	Abbreviations	Absolute Mass (*m/z*)	Ion/Reaction	Precursor Ion (m/z)	Product Ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)	Retention Time (min)
Pregnenolone-$^{13}C_2$-d_2	P_5-$^{13}C_2$-d_2	320.5	$[M+H-H_2O]^+$	303.6	161.3	40	5	40	10	13.36
Progesterone	P_4	314.5	$[M+H]^+$	315.1	97.2	75	15	25	10	19.21
Progesterone-$^{13}C_2$	P_4-$^{13}C_2$	316.5	$[M+H]^+$	317.6	99.1	75	10	45	15	19.18
Progesterone-$^{13}C_3$	P_4-$^{13}C_3$	317.5	$[M+H]^+$	318.4	112.2	50	10	35	5	19.18
Corticosteroids										
11-Deoxycorticosterone	DOC	330.5	$[M+H]^+$	331.7	109.3	75	10	35	5	17.10
11-Deoxycortisol	S	346.5	$[M+H]^+$	347.3	97.1	75	15	30	10	12.97
Corticosterone	B	346.5	$[M+H]^+$	347.2	121.3	40	5	20	15	13.18
Cortisol	F	362.5	$[M+H]^+$	363.1	121.1	75	10	25	10	11.36
Cortisol-d_4	F-d_4	366.5	$[M+H]^+$	367.4	121.3	75	10	25	10	11.32
Dansyl Chloride Derivatized Estrogens										
Dansyl Chloride Equilin	DC-EQ	268.4	Mono-DC	502.4	171.5	75	10	35	10	24.76
Dansyl Chloride Estradiol	DC-E_2	272.4	Mono-DC	506.4	171.5	125	5	35	15	19.42
Dansyl Chloride Estradiol-$^{13}C_3$	DC-E_2-$^{13}C_3$	275.4	Mono-DC	509.3	171.3	75	10	40	15	19.40
Dansyl Chloride Estriol	DC-E_3	288.4	Mono-DC	522.3	171.3	125	10	35	15	15.48
Dansyl Chloride Estrone	DC-E_1	270.4	Mono-DC	504.3	171.2	125	5	35	15	25.92

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Table 2

Limit of detection (LOD) and limit of quantitation (LOQ) for steroids measured in methanol, fetal bovine serum (FBS), and standards reference materials (SRMs). Statistical ($_{st}$) and empirical ($_{em}$) LODs were calculated and the highest value taken for the SRMs. Values are presented in total ng in the measured extract which was 2 mL of material.

Steroid	Internal Standard	Methanol		FBS		SRMs
		LOD_{st}	LOD_{em}	LOD_{st}	LOD_{em}	LOQ
11-Deoxycorticosterone	F-d_4	0.35	0.8	1.25	2	0.024
11-Deoxycortisol	F-d_4	0.29	0.8	1	2	3.05
17-OH-Pregnenolone	17-OH-P_4-$^{13}C_3$	0.23	0.8	0.68	0.16	[c] ND
17-OH-Progesterone	17-OH-P_4-$^{13}C_3$	[a] -	0.16	1.01	2	0.146
Adrenosterone	AE-$^{13}C_3$	0.051	0.06	0.94	2	1.92
Androstenedione	AE-$^{13}C_3$	0.12	0.06	1.05	2	0.0541
Corticosterone	F-d_4	-	0.16	[b] NA	>500	1.34
Cortisol	F-d_4	-	0.06	0.31	0.8	121
DC-Equilin	DC-E_2-$^{13}C_3$	0.36	0.16	0.043	0.8	ND
DC-Estradiol	DC-E_2-$^{13}C_3$	0.032	0.16	0.4	0.8	0.0156
DC-Estriol	DC-E_2-$^{13}C_3$	0.095	0.16	0.42	0.8	0.0197
DC-Estrone	DC-E_2-$^{13}C_3$	-	0.16	0.23	0.8	0.000570
DHEA	T -$^{13}C_3$	NA	>500	NA	4	ND
DHT	T -$^{13}C_3$	NA	10	NA	10	ND
Equilin	E_2-$^{13}C_3$	0.58	0.8	1.35	2	ND
Estradiol	E_2-$^{13}C_3$	0.64	0.8	1.09	2	ND
Estriol	E_2-$^{13}C_3$	6.33	10	NA	100	ND
Estrone	E_2-$^{13}C_3$	0.90	0.8	-	2	ND
Prenenolone	P_5-$^{13}C_2d_2$	0.26	0.8	0.66	0.8	ND
Progesterone	P_4-$^{13}C_2$	0.068	0.16	1.23	2	0.0894
Testosterone	T-$^{13}C_3$	-	0.06	0.8	2	0.140

[a] (–) negative value

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[b](NA) Could not be calculated

[c](ND) Not detected

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Table 3

Means and statistics for steroid hormone measurements in standard reference materials (SRMs). Extractions were performed on 2 mL of sample but values presented are mass adjusted to ng/g.

	SRM 1950 Plasma				SRM 971 M Male Serum				SRM 971 F Female Serum			
Steroid	**Mean (ng/g)**	**Stdev**	**RSD**	**Certified value (ng/g)**	**Mean (ng/g)**	**Stdev**	**RSD**	**Certified value (ng/g)**	**Mean (ng/g)**	**Stdev**	**RSD**	**Certified value (ng/g)**
DOC	0.0281	0.0069	24.5 %		0.0611	0.0410	67.1 %		0.0989	0.0599	60.6 %	
S	10.1	2.4	23.5 %		6.92	1.66	24.0 %		3.46	0.97	28.2 %	
17-OH-P4	1.19	0.06	4.88 %		1.80	0.05	2.77 %		1.26	0.20	16.2 %	
AT	0.398	0.040	10.1 %		0.365	0.020	5.38 %		0.252	0.042	16.9 %	
AE	0.817	0.008	1.03 %		0.543	0.015	2.82 %		0.658	0.034	5.11 %	
B	1.61	0.32	19.8 %		3.81	0.47	12.3 %		2.07	0.63	30.5 %	
F	93.9	6.05	6.44 %	82.2 ± [a]1.7	98.9	6.3	6.33 %	105.0 ± 1.5	80.7	9.2	11.4 %	88.5 ± 2.1
$DC\text{-}E_2$	0.0460	0.0029	6.28 %		[a]- <LOD	<LOD-	<LOD-		0.0881	0.0145	16.4 %	
$DC\text{-}E_3$	0.166	0.037	22.0 %		0.142	0.031	22.1 %		0.255	0.080	31.2 %	
$DC\text{-}E_1$	0.0394	0.0061	15.6 %		0.0310	0.0027	8.79 %		0.0723	0.0088	12.2 %	
P_4	1.44	0.01	0.595 %	1.452 ± 0.037	0.0403	0.0013	3.25 %	0.0403 ± 0.0062	1.96	0.13	6.60 %	1.903 ± 0.068
T	2.35	0.02	0.828 %	2.169 ± 0.046	5.89	0.33	5.63 %	6.279 ± 0.143	0.295	0.040	13.4 %	0.271 ± 0.006

[a] number following ± is the uncertainty