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Validation of a gas chromatography–mass spectrometry method for the determination of pg/ml levels of 17β-estradiol and 17β-trenbolone in bovine serum

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Abstract

A method for the quantitation of pg/ml levels of 17β-estradiol and 17β-trenbolone in bovine serum by gas chromatography/electron-capture mass spectrometry has been developed and validated. Using the area ratios of the integrated molecular-ion peaks of the analytes to their corresponding deuterated internal standards, [2,4,16,16-$^{2}H_4$] 17β-estradiol (17β-estradiol-d_4) and [16,16-$^{2}H_2$] 17β-trenbolone (17β-trenbolone-d_2), and non-weighted linear regression, two calibration curves per analyte; 5–50 and 50–500 pg/ml for 17β-estradiol in sera, and 25–250 and 250–2500 pg/ml for 17β-trenbolone in sera, respectively, were constructed. Splitless injection of 200 fg 17β-estradiol and 1000 fg 17β-trenbolone could be detected and quantified. Tested batches of control bovine sera did not exhibit interference for 17β-trenbolone, and showed expected background presence of endogenous 17β-estradiol. Intra-day residual errors did not exceed 20%, and regression correlations were greater than 0.99. Intra-day precision data was similar to inter-day precision data. Using this method, 16 samples can be processed within one working day.
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1. Introduction

Exposure to the pharmacologically active steroids 17β-estradiol and 17β-trenbolone can occur via pharmaceutical preparations, food consumption and the environment [1]. An endogenous hormone, 17β-estradiol has several uses in human health [2]. Both 17β-estradiol and 17β-trenbolone are used in animal husbandry practices; in combination they are potent growth-promoters [3]. When used appropriately, they have proven beneficial effects in human and veterinary medicine. However, inappropriate use of these compounds can result in negative effects, mediated primarily through endocrine disruption [1]. Therefore, the need to detect and quantify these compounds in various matrices is of interest from both agricultural and human health perspectives.

Because of their easy accessibility and utility as indicators of drug presence and concentration, sera and plasma are desirable matrices for analysis in research and development, pharmacokinetic studies, and chemical-residue monitoring programs. However, analytical methodologies for 17β-estradiol and 17β-trenbolone are particularly challenging to develop due to the sensitivity required; for 17β-estradiol analysis, analyte levels less than 10 pg/ml in plasma are common [3]. In addition to sensitivity, analytical methodologies must also meet predetermined values for specificity, accuracy, repeatability and reproducibility [4]. Traditionally, the analyses of these drugs were performed using immunological-based methods [5,6]. Though sensitive, these assays can not unambiguously assign a response to a given compound, and responses themselves are generally not reproducible, making quantitation suspect [7,8]. These characteristics do not make immunological methods readily applicable for studies requiring high accuracy and precision, and therefore a need to develop analytical methodology of comparable sensitivity but with superior specificity and reproducibility to immunoassays exists.

Chromatographic assays coupled to mass-spectrometric detectors offer multi-dimensional resolution of analytes from background interferences by chromatographic separation and molecular weight profiling of ions. A high level of confidence in the identification of compounds is attainable

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with this hybrid technology; Webb and Sargent [9] citing Burlingame et al. [10] and the LGC/VAM program [11] showed that using only the ratios of three characteristic ions within reasonable tolerance levels (±20% of peak ratios) unambiguous identification of compounds such as malathion and diethylstilbesterol in mass spectral databases was possible. It was further speculated by the authors that this criteria is applicable to other analytes.

Chromatographic–mass spectrometric assays have improved as a result of continual advances in related technologies. Traditionally, GC–MS assays were the predominant hyphenated technique for the analysis of steroids, primarily due to limitations in coupling and detection sensitivity in LC–MS applications [12]. However, GC–MS applications are limited to volatile steroids or steroids readily derivatized to volatile compounds, whereas the scope of compounds that are readily detectable by LC–MS is much broader; polar steroids as well as steroid conjugates can be analyzed without prior hydrolytic or derivatization reactions. Joos and Van Ryckeghem [13], using atmospheric pressure chemical ionization (APCI) in tandem mass spectrometry mode, obtained detection levels of less than 1 ng/g in kidney fat matrix for estradiol and trenbolone. Buiarelli et al. [14], also using APCI in tandem mass spectrometry mode, quantified trenbolone to 1 ng/g in bovine serum using 1 ml sample sizes. Electrospray ionization LC–MS–MS has also been reported to have excellent utility in analyzing structurally diverse anabolic steroids at low levels [15].

Despite the continuous advances in and obvious advantages of LC–MS techniques, sensitivity for certain applications remains insufficient. Depletion studies on 17β-estradiol and 17β-trenbolone in serum from heifers following implantation with commercially available growth-promoter formulations required analytical capabilities to less than 5 pg/ml for 17β-estradiol, and less than 25 pg/ml for 17β-trenbolone [16]. Research and development on the effects of ingestion of trace levels of endocrine disrupting compounds, including estradiol and trenbolone, on reproductive potential in animals is a growing concern [17]; it is assumed that such research would require analytical capabilities similar to that of pharmacokinetic studies. Other endocrinology studies would also be expected to have similar analytical requirements. For pharmacologically active compounds, chemical-residue monitoring programs require analytical methods with low limits of detection. To the best of the authors' knowledge, quantitation of 17β-estradiol and 17β-trenbolone to low pg/ml levels in serum or plasma is not currently attainable by LC–MS techniques when appropriate sample sizes are used.

However, gas chromatography coupled with mass-spectrometry has been used successfully to quantify low-levels of steroids in bodily fluids [18]. At the 48th American Society for Mass Spectrometry Conference, Bérubé et al. [19] presented data on the use of GC–MS for the analysis of estrogenic and androgenic steroids in rat and monkey sera. Liquid-liquid extraction using 1-chlorobutane followed by cleanup on normal-phase extraction cartridges was used to remove analytes from sera. The steroids were derivatized by either acylation with pentafluorobenzoyl chloride or oximation with pentafluorobenzoyl hydroxy amine. Using 0.4 ml serum samples, endogenous steroids in monkey and rat sera were reported to be quantifiable to the low pg/ml concentration using selected ion-monitoring of 1 or 2 ions per compound [19,20]. Though the method did not meet the requirements for quantitation and confirmation as suggested in the European Decision document 2002/657/EC [21], both accuracy and precision of the method were within 10%. Thus, Bérubé et al. demonstrated that gas chromatographic separation of derivatized analytes combined with selected-ion monitoring and pre-screening of matrix blanks was adequate for quantitation purposes.

There was a desire to apply this method to analyses in the authors' laboratory. However, for unknown reasons, application of this method to the analysis of 17β-estradiol and 17β-trenbolone in bovine sera showed limited success. The lack of success with 17β-trenbolone quantitation was expected, as this was the first known attempt to extract and derivatize the compound via this procedure. The lack of success with 17β-estradiol quantitation was unexpected because it was reported to be quantified by Bérubé et al. [19]. This showed that there was a need for method modification and development to satisfy analytical needs. Several extraction parameters (extraction solvent composition, serum volume, wash solvent composition, elution solvent composition) and derivatization parameters (amount of derivatizing agent added, derivatization solvent composition, derivatization time) were studied for their effects on quantitation of the analytes in question. A wide variability in results was collected early in the study, and subsequent testing was done according to the one-variable at a time approach to determine suitable values for method parameters. Using this approach, a method was developed that was then subjected to an in-house validation protocol to obtain estimates of intra- and inter-day accuracy and precision, selectivity, specificity, sensitivity and linearity. A description of the method modification procedure and the method validation statistics obtained are presented herein.

2. Experimental

2.1. Chemicals and reagents

1-Chlorobutane, ethyl acetate, hexane, methanol and pyridine, all distilled in glass, were obtained from Caledon Laboratories (Georgetown, Canada). Anhydrous sodium sulfate and potassium hydrogen carbonate, AnalaR grade, were obtained from VWR-Canlab (Toronto, Canada). Pentafluorobenzoyl chloride was obtained from Sigma–Aldrich (Mississauga, Canada). Reagent grade water was obtained from a NANOpure® water purification system (Fisher Scientific, Toronto, Canada).

17β-Trenbolone (purity 98.9%) was a gift from Hoechst Roussel Agrivet Company (Somerville, NJ, USA). 17β-Estradiol (purity > 98%) was obtained from Sigma–Aldrich. [2,4,16,16-$^2H_4$] 17β-Estradiol, (17β-estradiol-d_4), purity 99%, was purchased from C/D/N Isotopes (Pointe Claire, Canada). [16,16-$^2H_2$] 17β-Trenbolone, (17β-trenbolone-d_2), purity > 95%, was purchased from RIVM (Bilthoven, The Netherlands). All standards were stored at −20 °C until used.

Stock solutions of standards in methanol (100 μg/ml) were stored at −20°C for up to 6 months (17β-trenbolone and 17β-trenbolone-d_2) or 1 year (17β-estradiol and 17β-estradiol-d_4). Hydrogen/deuterium (H/D) back exchange of the deuterated standards was not expected to occur in solution to any significant extent at low temperatures and in the absence of acid catalyst. The absence of significant H/D back exchange was suggested by the lack of significant changes in analyte/deuterated-analyte ratios at all tested concentrations as a function of time; this was determined by regression analysis on time and obtained ratios (independent and dependent variables, respectively). Regression statistics showed no trends ($n = 9$, $P > 0.05$), thereby suggesting that significant H/D back-exchange in solution did not occur during the time course of the validation. As well, full scan mass spectra of deuterated standards did not show the presence of protonated analogs, implying that ion source propagated back exchange did not occur.

Mixed working standard solutions of 17β-estradiol and 17β-trenbolone in methanol were 0.5–500 and 0.10–100 ng/g, respectively. The internal standard solution, stored at −20 °C until used, was 1 ng/g of 17β-estradiol-d_4 and 500 ng/g of 17β-trenbolone-d_2 in methanol.

2.2. Matrix standards

Negative-control bovine sera were a gift from CAVL (Amarillo, TX, USA) and were analyzed prior to use to verify the absence of 17β-trenbolone. Using matrix over spikes, 17β-estradiol concentration in all sera samples was determined to be less than 5 pg/ml. Frozen sera were thawed at room temperature, mixed gently, and 2 ml aliquots were placed into 15 ml glass centrifuge tubes. The aliquots were spiked with 100 μl of appropriate mixed working standard solutions and 100 μl of mixed deuterated internal standards to obtain matrix standards with 17β-estradiol and 17β-trenbolone concentrations of 5–500 and 25–2500 pg/ml, respectively, and 17β-estradiol-d_4 and 17β-trenbolone-d_2 concentrations of 50 and 25 ng/g, respectively. 17β-Estradiol-d_4 and 17β-trenbolone-d_2 concentrations were chosen so that the maximum contribution from the 17β-estradiol [$M+4$]-isotope and 17β-trenbolone [$M+2$]-isotope would not exceed 1% of the concentration of deuterated compounds. The [$M+4$]-contribution for derivatized 17β-estradiol is 0.1% of base mass; the [$M+2$]-contribution for derivatized 17β-trenbolone is 4.2%. With these contributions, 500 pg/ml 17β-estradiol contributed 0.5 pg/ml [$M+4$]-; and 2500 pg/ml 17β-trenbolone contributed 105 pg/ml [$M+2$]-, both less than 1% of their corresponding deuterated internal standard [M]-concentrations.

2.3. Extraction and derivatization of analytes

Analytes were extracted from 2 ml serum aliquots by liquid–liquid extraction using 1-chlorobutane (2 ml × 3). The 1-chlorobutane extracts were then applied to Bond-Elut silica gel cartridges (500 mg, 3 ml), purchased from Varian (Harbor City, CA, USA) and partially deactivated by washing with three-column volumes (3 ml × 3) of water-saturated ethyl acetate. After extract application, the cartridges were washed sequentially with one column volume of hexane and hexane–ethyl acetate (90:10), and eluted with two column volumes of 50:50 hexane–ethyl acetate into 15 ml glass centrifuge tubes. The organic phases were removed under a gentle stream of N_2 at room temperature.

The dried extracts were derivatized using 500 μl of a 10% solution of pyridine in dry ethyl acetate, and 100 μl of a 5% solution of pentafluorobenzoyl chloride in dry ethyl-acetate in sealed glass centrifuge tubes for 20 min at 60 °C. Solvents were then removed under a gentle stream of N_2 at room temperature, and 2 ml of 0.5 M $KHCO_3$ was added to all tubes to deactivate any remaining derivatizing agent. The aqueous phases were extracted with hexane (2 ml × 3). The solvents were removed under a gentle stream of N_2 at room temperature, and the residues dissolved in 100 μl of *iso*-octane prior to analysis.

2.4. GC–MSD analysis

An Agilent 6890 GC with a 5973N MSD and Chemstation analytical software was used for analysis. A DB-5MS column (J and W Scientific, 30 m × 0.25 mm × 0.25 μm film thickness) was used under the following conditions: carrier gas: He, 1.0 ml/min for 5 min, then ramp 0.5 ml/min to 3 ml/min; oven ramp: 195 °C for 1.5 min, then ramp 20 °C/min to 270 °C, hold 8 min, then ramp 15 °C/min to 290 °C, hold 5 min, then ramp 10 °C/min to 300 °C, hold 5 min, then ramp 10 °C/min to 305 °C; injection: 2 μl, pulsed splitless injection, 900 μl splitless single-taper liner (Agilent part # 5181-3316), 300 °C, 30 psi for 1 min.

Quantitation was performed using the chemical ionisation source with Chemstation analytical software under the following conditions: MS interface, 300 °C; MS source, 150 °C (default); MS quadrupoles, 106 °C (default); ionization: electron capture mass spectrometry using methane as buffer gas, gas controller setting, 40; detection: selected-ion monitoring, *m/z* 464.2, 17β-trenbolone; *m/z* 466.2, 17β-trenbolone-d_2; *m/z* 660.3, 17β-estradiol; *m/z* 664.2, 17β-estradiol-d_4; dwell times 100 ms, electron multiplier setting, 400 eV above autotune.

Calculations were performed using the relative ratios of the peak areas of analytes to their deuterated analogs as a function of analyte concentration. A minimum of five points (blank not included) was used for calculating regression curves.

3. Results and discussion

3.1. Extraction

Being only moderately polar, free steroids can be easily extracted from aqueous media using apolar solvents or solid-phase media of varying chemistries [22]. However, steroid quantitation may be hindered by co-extraction of other endogenous components, which are usually present at much higher concentrations in the matrix than the analytes of interest [23]. Conditions must be established that selectively extract the analytes while minimizing co-extraction of interferences. Immunosorbent technology is regarded as being the most selective extraction mechanism; disadvantages of this technology are its high cost relative to other SPE mechanisms, and the fact that such specificity may not be desirable when performing multi-analyte analyses [22]. When applied to studies requiring numerous analyses, these disadvantages outweigh their superior selective properties. Reversed-phase SPE devices are the most popular devices for removal of analytes from biomatrices [22]. However, being based primarily on hydrophobic interactions with secondary polar interactions, they are not selective enough when used as the sole extraction criteria. For steroid analyses, several different extraction mechanisms and steps may be required to achieve a desired degree of cleanup [24,25].

However, it should be noted that for each additional extraction step used, precision of the method decreases; Tölgyessy and Liška [26] showed that the relative standard deviation (R.S.D.) at each processing step that used LLE and SPE extraction chemistries for several model compounds ranged from 10 to 30%. Because losses in precision are additive, the final relative standard deviation in a process can be substantial. Reducing the number of processing steps may not be possible. It is for these reasons that internal standards are included in methodologies; variations introduced at each step for internal standard and compound will be partially correlated with each other, thereby reducing the final R.S.D. Isotopically labeled analogues of the analytes of interest are generally regarded as having nearly identical chemical properties as their non-labelled counterpart, and therefore having greater correlation.

In the Bérubé et al. method [19], the extraction strategy used a minimum number of processing steps as well as incorporation of deuterated internal standards to increase precision. The use of chlorobutane as an initial extraction solvent may have aided in reducing the number of processing steps; while chlorobutane has the solvating power of a chlorinated solvent, its four-carbon chain reduces its polarity. As a result, polar compounds will be left in the aqueous phase, while apolar or moderately polar compounds are extracted. Other organic solvents may not be as selective. The extract was then cleaned with silica gel cartridges. Silica and other normal-phase materials are used in chromatography to separate lipid materials into different classes [27]. After judicious use of elution solvents, Bérubé et al. [19] obtained extracts that upon analysis produced relatively clean chromatograms.

However, it was found that using the unmodified method gave poor, variable recoveries. It was determined that the poor recovery was due to strong adsorption of the analytes in the silica SPE cartridges. Eluting cartridges with methanol or acetone did not increase recoveries. It was therefore decided to determine the effect of deactivating the cartridges on recoveries. Deactivation effects on recovery were assessed by passing water saturated ethyl–acetate through the columns, while still using the same wash (10–90 ethyl acetate–hexane) and elution (50–50 ethyl acetate–hexane) solvents. It was found that both analyte recovery and background noise increased. Three column volumes of water-saturated ethyl acetate were found to provide suitable recovery and background noise.

Because of environmental concerns, non-chlorinated solvents were evaluated for extraction efficacy. Ethyl ether and ethyl acetate extracts resulted in good recoveries (>80%), but caused extensive ion-source fouling, resulting in frequent mass spectrometer downtime. Hexane extractions gave poor recoveries (<20%). It was decided to continue using 1-chlorobutane as the initial extraction solvent.

The serum volume required in this method was higher than that required by the original method (0.4 ml). Increasing serum volume increased response, but also increased background noise. Suitable signal strength for chromatographic peaks (s/n > 10, as determined by Chemstation software) of 5 and 25 pg/ml sera spikes of 17β-estradiol and 17β-trenbolone, respectively, was obtained using 2 ml serum.

Wash and elution solvent compositions were varied by changing the relative compositions of hexane and ethyl acetate. It was found that using solvent proportions between the suggested 90–10 and 50–50 hexane–ethyl acetate for wash and elution solvents respectively (20–80 and 40–60) would result in either partial elution of analytes during washing, or incomplete elution of analytes in the final step. Bérubé's et al. [19] solvent compositions were therefore used.

3.2. Derivatization

Pentafluorobenzoyl derivatives have been used with success in quantitative gas chromatographic assays. Compounds with accessible, acidic hydrogens can be derivatized using pentafluorobenzoyl chloride as the derivatizing agent. Early work done by other researchers using this derivative in conjunction with electron-capture detectors showed that diethylstilbestrol, dienestrol and hexestrol residues could be detected at sub ng/g levels [28]. Pentafluorobenzoyl

derivatives have been reported to be superior to other fluorinated derivatives with regards to forming abundant molecular ions during negative-ion chemical ionization [29,30]. When selected ion-monitoring of these molecular ions is combined with the further discriminating power of capillary gas chromatography, detection and quantitation of trace levels of steroids is possible.

Derivatization conditions were found to have variable effects on analyte quantitation. Container type had a large impact; polypropylene tubes contributed larger and more numerous interfering contaminating peaks than glass tubes. Silanized glass tubes did not increase recoveries relative to non-silanized glass tubes ($n = 3$, $P > 0.05$), and therefore non-silanized tubes were used for subsequent testing. The absence or presence of base catalysts (pyridine or sodium carbonate) in the derivatizing mixture did not show identifiable differences ($n = 3$, $P > 0.05$). However, because the acylation reaction is base catalyzed, it was decided that pyridine would be used in all subsequent derivatizing solutions as was done by Bérubé et al. [19]. Varying derivatization time between 5 and 30 min did not show significant quantitative differences ($n = 3$, $P > 0.05$) though 20 min derivatization time had the lowest variability, and was subsequently used afterwards; the method of Bérubé et al. used a 30 min derivatization time. Deactivation of remaining derivatizing agent with $KHCO_3$ was visibly observed to be nearly instantaneous; contrasting with Bérubé's et al. 15 min waiting period. Therefore, the 15 min waiting period was eliminated from the final method.

Mass spectra of the derivatized standards (2 ng injected) revealed one predominant base ion for each compound; *m/z* 660.3 for 17β-estradiol, and *m/z* 464.2 for 17β-trenbolone. The derivatized estradiol spectrum contained an additional, small (ca. 10%) *m/z* 448 ion. Given that the mass spectrometer used (Agilent 5973) operated in the negative chemical ionization mode as an electron capture mass spectrometer, simple spectra were expected. The 17β-estradiol spectra contrasted with the spectrum reported by both Bérubé et al. [19] and Xiao et al. [30]; both showed that the *m/z* 448 ion was approximately 50% of the base ion; and Xiao showed an additional significant *m/z* 148 ion. Such differences can be attributed to the use of different mass spectrometric conditions. Bérubé et al. did not reveal the mass spectrometric conditions used in their study [19], therefore, a comparison among conditions used to obtain spectra by the different methods was not possible.

The lack of fragment ions indicates that this method cannot be considered for confirmatory purposes in chemical-residue monitoring programs as outlined in 2002/EC/657 [21]. However, no substantial interferences in the chromatograms of several processed sera were noted (see representative chromatograms, Figs. 1 and 2), indicating that the method can be used as a screening method in chemical-residue monitoring programs, and as a quantitative method when definable populations of matrices (research and development, pharmacokinetics) are studied.

3.3. Method statistics

Figs. 1 and 2 illustrate the sensitivity of the method, and show that 5 and 25 pg/ml of 17β-estradiol and 17β-trenbolone in serum are readily detected (approximately 200 and 1000 fg injected, respectively), and that co-extractants did not interfere significantly with the chromatographic resolution and detection of the compounds. Analyte recoveries exceeded 90% at all concentrations. Bérubé et al. [19] reported detection of 30 fg estradiol from extracted serum injected which, as suggested previously, may be due to different mass spectrometric conditions. Xiao et al. [30] reported detection of 0.2 ng/l estradiol in river water, based on a 2.5 l sample. A comparison of Xiao's method with the present and Berube's method would not be valid due to the use of different matrices, equipment, and ionization conditions, but the high sensitivities of all methods are evident.

Method sensitivity can be maintained for approximately 100 injections prior to cleaning the ion-source. Chromatographic resolution of the eluted compounds is more robust; the number of injections that this resolution is maintained can exceed 500 injections.

CC-alpha and CC-beta values for each analyte in the method were calculated based on data acquired during the validation procedure from the three lowest calibration

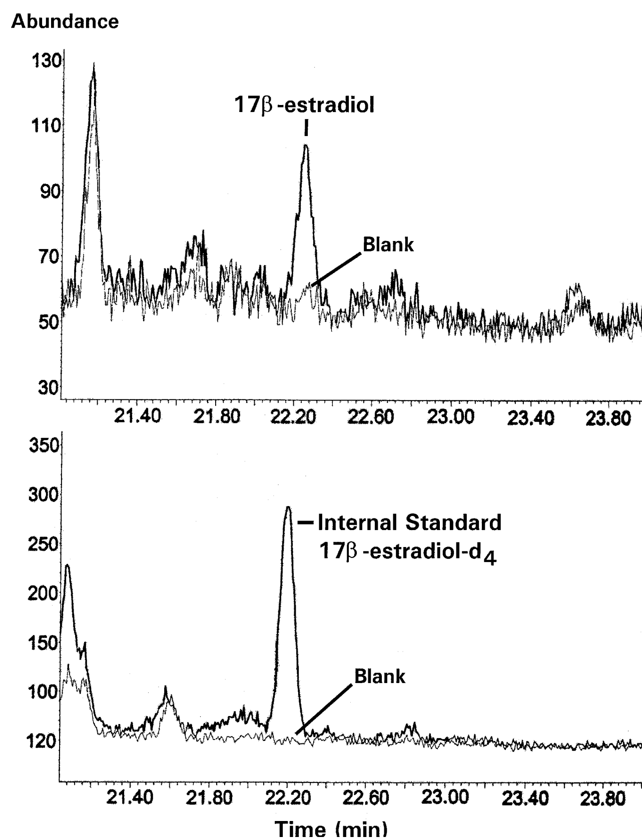

Fig. 1. Representative selected-ion monitoring chromatograms of 17β-estradiol, 5 pg/ml, *m/z* 660.2; and 17β-estradiol-d_4, 50 pg/ml, *m/z* 664.2, extracted from bovine serum.

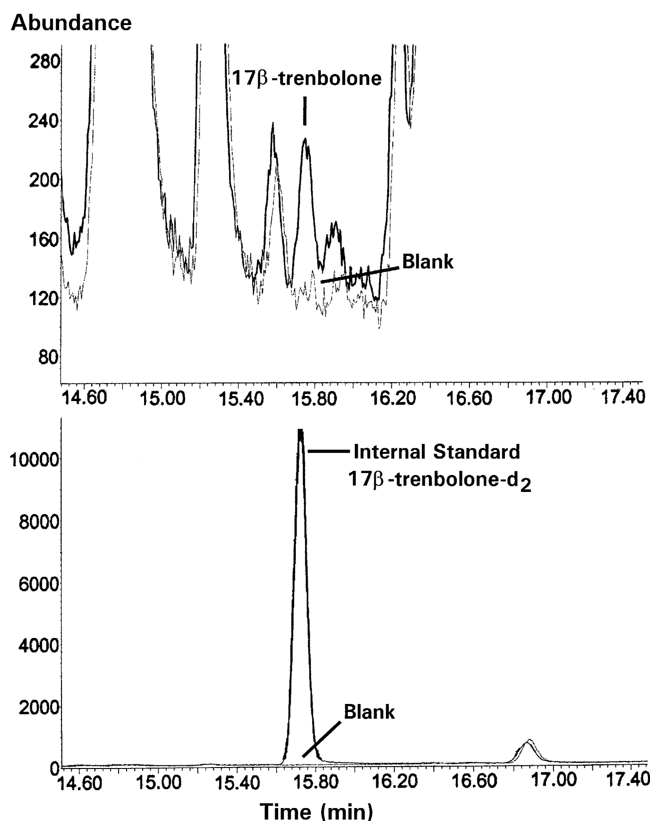

Fig. 2. Representative selected-ion monitoring chromatograms of 17β-trenbolone, 25 pg/ml, m/z 464.2; and 17β-trenbolone-d_2, 25 ng/ml, m/z 466.2, extracted from bovine serum.

standards. The three lowest calibration standards were used as they satisfy the requirement for homeogeneity of variances. Using this protocol, CC-alpha and CC-beta values for 17β-estradiol and 17β-trenbolone were calculated to be 10.3 and 17.3 pg/ml, and 49.3 and 82.7 pg/ml, respectively.

The method's intra-day accuracy and precision estimates are shown in Table 1. For both analytes over the entire analytical range, R.S.D. did not exceed 12%. Inaccuracy of 17β-trenbolone quantitation decreased as analyte concentration increased, whereas inaccuracy of β-estradiol quantitation appeared to be independent of concentration.

The method's inter-day accuracy and precision estimates, which show matrix and time effects [31], were collected over a period of 9–11 runs and are shown in Table 2. As expected, the greatest R.S.D. and deviations from expected occurred at the lowest concentrations (inter-day variability). However, inaccuracy and R.S.D. did not exceed 15%, indicating that time did not appear to decrease accuracy or precision of the method. These statistics compared favorably to those obtained by Bérubé et al. (inter assay R.S.D. $< 8\%$). Xiao et al. [30] used the same derivatizing reagent and quantified 10 ng/l β-estradiol in 2.5 l of ground water and effluents with an R.S.D. ranging from 2.6 to 9.8%.

The current method, Bérubé's, and Xiao's method used deuterated analogs of the analytes as internal standards, and were added prior to extraction. Since deuterated analogs have almost identical chemistry to the analytes, their extraction, derivatization, chromatography and detection should be highly correlated to that of the analytes. During the course of the study, it was determined that at all concentrations correlations between area counts for estradiol and its deuterated analog exceeded 0.98, whereas correlations between area counts for trenbolone and its deuterated analog exceeded 0.995. The higher degree of correlation seen between trenbolone and its deuterated analog may be due to the fact that the trenbolone internal standard was present at a much higher concentration than the estradiol internal standard, and therefore its quantitation would be less affected by method variation. Correlation between area counts for the two internal standards was less than 0.94, indicating that method conditions affecting quantitation were compound dependent. Calibration line correlation coefficients exceeded 0.993 for both compounds on all test runs.

Good reproducibility (inter-day) of the estimated calibration slopes was obtained. For 17β-estradiol, inter-day R.S.D.s of the slopes for the 5–50 and 50–500 pg/ml regressions were 17.1 and 16.9%, respectively. For β-trenbolone, inter-day variation of the slopes for the 25–250 and 250–2500 pg/ml regressions were 8.21 and 2.45%, respectively. The reason for the increased variability in the β-estradiol regression statistics was not investigated further; though it was speculated to be due to the lower correlation

Table 1
Intra-day inaccuracy and repeatability for 17β-estradiol and 17β-trenbolone

Nominal concentration estradiol; trenbolone (pg/ml)	Parameter[a]		17β-Estradiol	17β-Trenbolone
5.00; 25.0	$n = 6$	Mean (pg/ml)	4.83	31.6
		CV (%)	11.5	11
		Inaccuracy (%)	−3.4	26.4
50.0; 250	$n = 6$	Mean (pg/ml)	52.7	272
		CV (%)	3.3	2.5
		Inaccuracy (%)	5.4	8.6
500; 2500	$n = 6$	Mean (pg/ml)	474	2400
		CV (%)	12	3.1
		Inaccuracy (%)	−5.2	−4.2

[a] From cumulative data, 3 runs.

Table 2
Inter-day accuracy and repeatability for 17β-estradiol and 17β-trenbolone

Nominal concentration, estradiol; trenbolone (pg/ml)	Parameter[a]		17β-Estradiol	17β-Trenbolone
5.00; 25.0	$n = 10$	Mean (pg/ml)	5.51	25.4
		CV (%)	9.01	11.2
		Inaccuracy (%)	10.2	1.76
12.5; 62.5	$n = 10$	Mean (pg/ml)	12.3	64
		CV (%)	3.66	4.56
		Inaccuracy (%)	−1.36	2.44
25.0; 125	$n = 10$	Mean (pg/ml)	24.5	122
		CV (%)	4.64	6.68
		Inaccuracy (%)	−2	−2.08
40.0; 200	$n = 10$	Mean (pg/ml)	39.6	199
		CV (%)	4	4.82
		Inaccuracy (%)	−1.13	−0.6
50.0; 250	$n = 11$	Mean (pg/ml)	50.6	252
		CV (%)	2.01	2.29
		Inaccuracy (%)	1.2	0.73
125; 625	$n = 9$	Mean (pg/ml)	126	627
		CV (%)	2.5	2.16
		Inaccuracy (%)	0.76	0.32
200; 1000	$n = 9$	Mean (pg/ml)	200	998
		CV (%)	3.04	2.72
		Inaccuracy (%)	0.07	−0.24
250; 1250	$n = 9$	Mean (pg/ml)	244	1240
		CV (%)	2.64	3.16
		Inaccuracy (%)	−2.56	−0.77
500; 2500	$n = 9$	Mean (pg/ml)	503	2530
		CV (%)	0.48	1.11
		Inaccuracy (%)	0.53	1.09

[a] From cumulative data, runs 1–11.

seen between estradiol and its deuterated internal standard. The lower correlation may be due to H/D back exchange, however, testing indicated that in solution, this was not statistically significant ($P < 0.05$, details in materials and methods section). Ion-source mediated H/D exchange may have been another source of increased error, but no formal experiments were performed to verify this.

For 17β-estradiol, inter-day R.S.D.s of the intercepts for the 5–50 and 50–500 pg/ml regressions were 53.4 and 54.2%, respectively. For 17β-trenbolone, inter-day variation of the intercepts for the 25–250 and 250–2500 pg/ml regressions were 46.4 and 201%, respectively. The 95% confidence intervals (high-range curves) were 2.99 ± 1.65 pg/ml, for 17β-estradiol, and 13.7 ± 23.4 pg/ml for 17β-trenbolone. The estradiol regression intercept was significantly different from zero, suggesting that endogenous compound is present in the sera, which was expected. Trenbolone regressions showed that intercepts were not significantly different from zero. Since trenbolone is not an endogenous hormone, and the sera were obtained from a known source, the lack of a significant intercept in the regression was expected.

Stability studies of the analytes in serum over a 4-week period storage at −20 °C and two freeze-thaw cycles did not show any significant changes in concentrations over the full calibration range. Data obtained from blind spikes (Table 3) showed that accuracy of the method exceeded requirements, with the greatest errors being less than 12% from expected.

Figs. 3 and 4 show absolute residuals of the data collected over all runs as a function of concentration for 17β-estradiol and 17β-trenbolone, respectively. Because these data were a function of concentration, the standard fan-shape variation

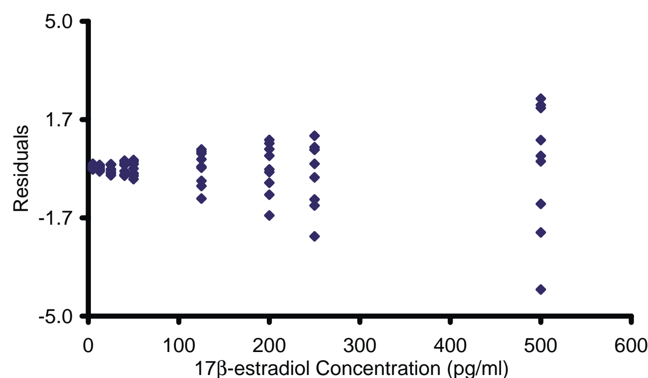

Fig. 3. Method performance: 17β-estradiol residuals.

Table 3
Blind spike analyses

Nominal concentration estradiol; trenbolone (pg/ml)	Parameter	17β-Estradiol	17β-Trenbolone
11.00; 2250.00	Calculated (pg/ml)	10.8	2420
	Error (%)	−1.58	7.5
11.00; 2250.00	Calculated (pg/ml)	11.3	2530
	Error (%)	2.45	12.4
300.00; 125.00	Calculated (pg/ml)	297	118
	Error (%)	−1.07	−5.46
108.75; 125.00	Calculated (pg/ml)	110	122
	Error (%)	1.02	−2.62
300.00; 66.25	Calculated (pg/ml)	289	60.2
	Error (%)	−3.5	−9.21
108.75; 66.25	Calculated (pg/ml)	114	67.3
	Error (%)	4.46	1.63

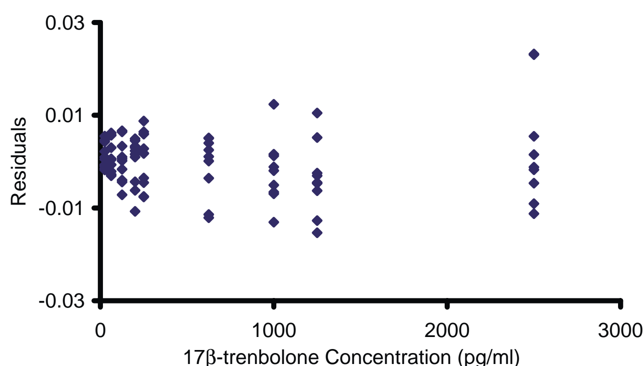

Fig. 4. Method performance: 17β-trenbolone residuals.

of the residuals was obtained. The 17β-trenbolone residuals appeared to have a more homogeneous variation as a function of concentration, whereas the 17β-estradiol residuals showed increasing variability with increasing concentration. Given that the 17β-trenbolone residual variances as a function of concentration were more homogenous, it can be assumed that its quantitation was relatively more independent of time and concentration effects than 17β-estradiol. In contrast, 17β-estradiol quantitation was dramatically affected, indicating that method performance with regards to measurement changed over time. Time is also a function of several factors, including extraction, derivatization, chromatography and detection; each factor itself is also a function of other variables. However, the use of calibration samples for each run would satisfy requirements for within-run accuracy and precision.

4. Conclusions

A validated method using GC–MS with electron-capture mass spectrometry and deuterated internal standards has been shown to be a sensitive and precise procedure for quantifying 17β-estradiol and 17β-trenbolone to low pg/ml concentrations in bovine serum. The low-cost instrumentation used indicates that this method can be performed by most reasonably equipped laboratories. A two step extraction-cleaning procedure was shown to be adequate to quantitatively remove the analytes from serum while minimizing co-extraction of interferences. In conjunction with capillary chromatography and selected-ion monitoring of molecular ions, 17β-estradiol and 17β-trenbolone could be quantified at concentrations of 5–500 and 25–2500 pg/ml, respectively, in 2 ml sera, with accuracies of 80–120% and variability less than 15%.

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