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Accurate and sensitive liquid chromatography/tandem mass spectrometry simultaneous assay of seven steroids in monkey brain

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ABSTRACT

Background: Following its secretion mainly by the adrenal glands, dehydroepiandrosterone (DHEA) acts primarily in the cells/tissues which express the enzymes catalyzing its intracellular conversion into sex steroids by the mechanisms of intracrinology. Although reliable assays of endogenous serum steroids are now available using mass spectrometry (MS)-based technology, sample preparation from tissue matrices remains a challenge. This is especially the case with high lipid-containing tissues such as the brain. With the combination of a UPLC system with a sensitive tandem MS, it is now possible to measure endogenous unconjugated steroids in monkey brain tissue.

Methods: A Shimadzu UPLC LC-30AD system coupled to a tandem MS AB Sciex Qtrap 6500 system was used.

Results: The lower limits of quantifications are achieved at 250 pg/mL for DHEA, 200 pg/mL for 5-androstenediol (5-diol), 12 pg/mL for androstenedione (4-dione), 50 pg/mL for testosterone (Testo), 10 pg/mL for dihydrotestosterone (DHT), 4 pg/mL for estrone (E_1) and 1 pg/mL for estradiol (E_2). The linearity and accuracy of quality controls (QCs) and endogenous quality controls (EndoQCs) are according to the guidelines of the regulatory agencies for all seven compounds.

Conclusion: We describe a highly sensitive, specific and robust LC–MS/MS method for the simultaneous measurement of seven unconjugated steroids in monkey brain tissue. The single and small amount of sample required using a relatively simple preparation method should be useful for steroid assays in various peripheral tissues and thus help analysis of the role of locally-made sex steroids in the regulation of specific physiological functions.

1. Introduction

In the human, DHEA sulfate together with unconjugated DHEA circulate in the blood as the largest amount of the entire spectrum of steroids with peak levels reached by the age of 25–30 years, followed by a gradual decrease at later age [1,2]. Since there is no other significant source of sex steroids after menopause [1,3], the decline in DHEA-derived sex steroids is likely involved, combined with the aging process, in a series of medical problems potentially including memory loss, cognition loss and Alzheimer's disease [4–8].

In addition to the steroids such as DHEA which may access the brain from the peripheral circulation, steroids can be synthesized/metabolized locally within the brain [9–16] Most interestingly, it has been reported that neurosteroids exert protective effects in several models of neurodegenerescence [17–20].

A wealth of evidence cumulated over the years indicates that DHEA and its active metabolites could play a key role in neurocognitive functions. A crucial need thus exists to precisely and accurately measure unconjugated steroids in brain tissue to better evaluate the importance of neurosteroids in cognitive functions and possibly cognitive impairment. The monkey appears as a particularly useful model since the higher primates are most closely related to humans in terms of anatomy and steroidogenesis than any other animal model [21,22]. Moreover, the use of monkey brain material permits to obtain an accurate measure of neurosteroid concentrations by avoiding delays between blood collection and tissue sampling.

The assay of hydroxysteroids in serum, plasma, urine and saliva has been developed [23–28]. However, measurement of tissue steroid levels is subject to additional methodological complexities [29]. Gas chromatography–mass spectrometry (GC–MS) alone as well as immuno-based assays have been the techniques used [30–34]. In the clinical laboratory, the conventional technique is still based upon immunoassays. The specificity of immuno-based

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assays is highly questionable and their sensitivity does not meet the requirement for many compounds [35–38]. GC–MS/MS, on the other hand, has encountered robustness issues.

Although earlier studies on brain steroid concentrations analyzed by RIA or GC–MS have shown relatively high levels for these compounds, other reports based on more direct measurement using a LC–MS/MS method found substantially lower concentrations in brain tissue [9,39] [40–44]. The challenge for the LC–MS/MS quantification of unconjugated steroids rests on sensitivity and specificity. The concentration of many endogenous steroids is at the lower pg/mL level [38] and many steroids have weak ionization efficiency due to their neutral nature. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has been reported for the measurement of steroids with or without derivatization [29,45–48].

Most available steroid assays have been done using rats or mice. However, the adrenal glands in species below primates do not secrete DHEA in significant amounts [49]. Apart from the highest homology of the steroidogenic enzymes between humans and monkeys [21,22], brain steroids of these higher primates are likely to be the sum of the contribution of unmodified or modified products of the classical endocrine glands (gonads and adrenals) added the steroids derived from circulating precursor steroids such as DHEA, or even synthesized from cholesterol locally, thus illustrating the need for simultaneous, accurate and precise measurement of many steroids.

Recently, our group has developed a fully validated robust and sensitive validated LC–MS/MS method to quantify seven endogenous steroids in a single serum sample according to the FDA regulatory guidelines [50] for unconjugated steroids with a LLOQ of 500 pg/mL for DHEA, 100 pg/mL for 5-diol, 100 pg/mL for 4-dione, 50 pg/mL for Testo, 10 pg/mL for DHT, 4 pg/mL for E_1 and 1 pg/mL for E_2 [29]. However, there is a need for a reliable method to quantify all these steroids from a small brain sample.

In the present report, we describe a novel robust, specific and sensitive LC–MS/MS method for the simultaneous quantification of seven steroidal compounds, namely DHEA, 5-diol, 4-dione, Testo, DHT, E_2 and E_1 using a single brain sample and simple preparation which ensures the efficient, accurate and valid measurement of unconjugated steroids at low endogenous levels in brain tissue.

2. Material and methods

2.1. Animals and tissue

All animals have been maintained and handled in accordance with the policies of the Canadian Council on Animal Care and the National Institutes of Health Guide for the Care and Use of Laboratory animals. Animals were perfused with a ice-cold saline solution prior to male cynomolgus monkey brain collection and immediate freezing in liquid nitrogen.

2.2. Chemicals

Dansyl chloride ($\geqslant$99.0%) and ammonium acetate (99.999% in metal basis) were purchased from Sigma (Oakville, Canada). High purity methanol (LC–MS grade), acetone, 1-chlorobutane and formic acid (88% GR ACS) were from EMD (Mississauga, Canada). Sodium bicarbonate was bought from Avantor, a brand from J.T. Baker (Center Valley, PA, USA). Reagent alcohol was from Fisher

Table 1
Working and spiking solutions for all seven calibrants and their quality controls as well as endogenous quality controls.

ng/mL DHEA	ng/mL 5-diol	ng/mL 4-dione	ng/mL TESTO	ng/mL DHT	ng/ml E_1	ng/mL E_2	ID
(A) Resulting working solutions							
25	20	1.2	5	1	0.4	0.1	WSTDB
50	40	2.4	10	2	0.8	0.2	WSTDC
200	160	9.6	40	8	3.2	0.8	WSTDD
750	600	36	150	30	12	3	WSTDE
2000	1600	96	400	80	32	8	WSTDF
3000	2400	144	600	120	48	12	WSTDG
4000	3200	192	800	160	64	16	WSTDH
5000	4000	240	1000	200	80	20	WSTDI
25	20	1.2	5	1	0.4	0.1	WLLOQC
75	60	3.6	15	3	1.2	0.3	WLowQC
2500	2000	120	500	100	40	10	WMedQC
3750	3000	180	750	150	60	15	WHighQC
pg/mL DHEA	pg/mL 5-diol	pg/mL 4-dione	pg/mL TESTO	pg/mL DHT	pg/ml E_1	pg/mL E_2	ID
(B) Resulting STD curve matrix samples							
250	200	12	50	10	4	1	STDB
500	400	24	100	20	8	2	STDC
2000	1600	96	400	80	32	8	STDD
7500	6000	360	1500	300	120	30	STDE
20,000	16,000	960	4000	800	320	80	STDF
30,000	24,000	1440	6000	1200	480	120	STDG
40,000	32,000	1920	8000	1600	640	160	STDH
50,000	40,000	2400	10,000	2000	800	200	STDI
250	200	12	50	10	4	1	LLOQC
750	600	36	150	30	12	3	LowQC
25,000	20,000	1200	5000	1000	400	100	MedQC
37,500	30,000	1800	7500	1500	600	150	HighQC
(C) Resulting tissue matrix samples							
X	X	X	X	X	X	X	EndoQC
750 + X	600 + X	36 + X	150 + X	30 + X	12 + X	3 + X	EndoLowQC
25,000 + X	20,000 + X	1200 + X	5000 + X	1000 + X	400 + X	100 + X	EndoMedQC
37,500 + X	30,000 + X	1800 + X	7500 + X	1500 + X	600 + X	150 + X	EndoHighQC

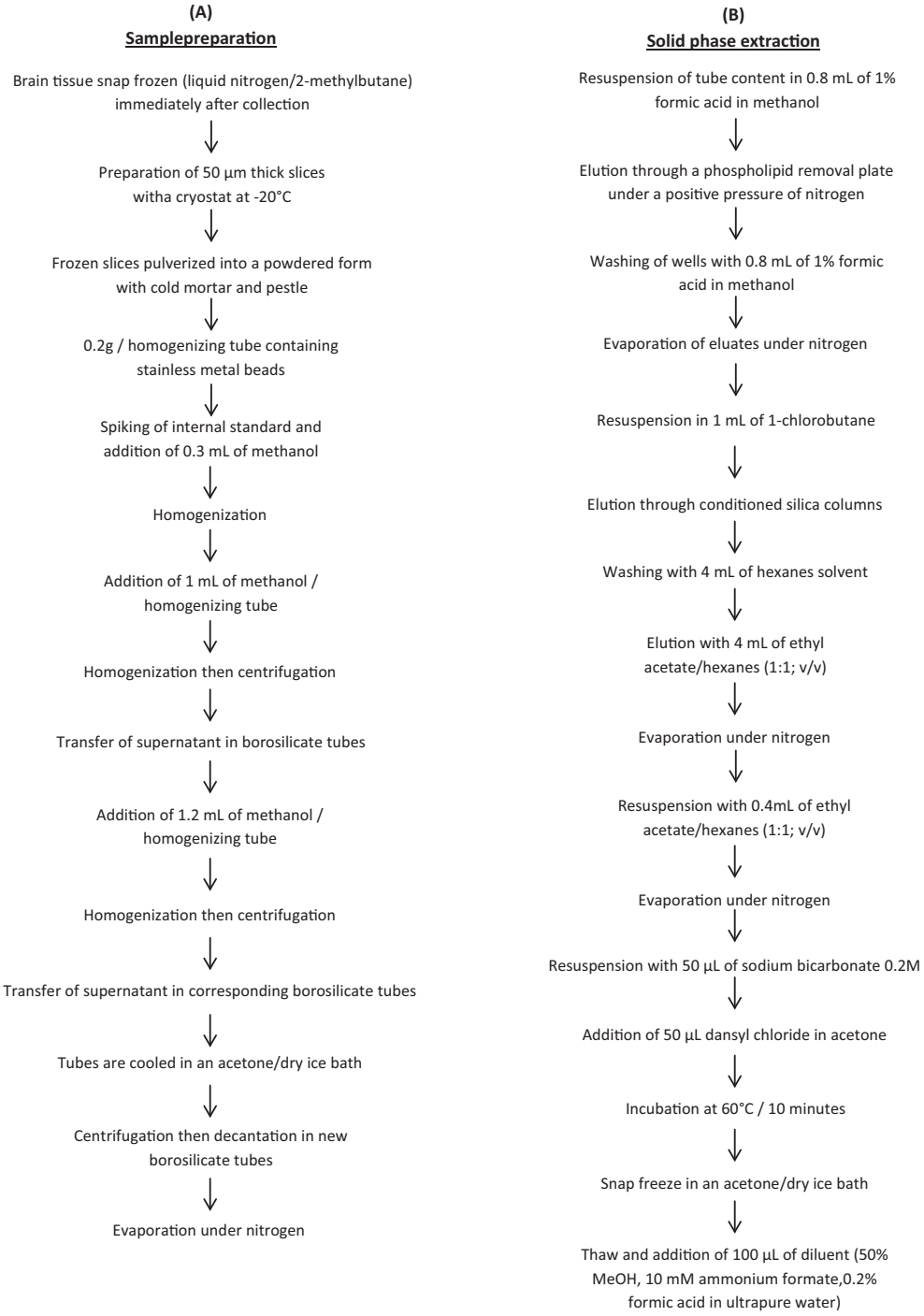

Fig. 1. Schematic representation of the overall sample preparation for injection on LC-MS/MS.

Scientific (Ottawa, Canada), while bovine serum albumin (BSA) was obtained from Ameresco (OH, USA). Ultrapure water was provided in house with a Millipore system.

2.3. Stocks, standards and quality control preparations

Stock solutions were prepared in reagent grade alcohol, while intermediate and working solutions were diluted in methanol. The calibration curve of each compound includes eight calibrants while its quality controls (QC) include four levels (Table 1). The calibration curves and the quality controls were prepared in ultrapure water containing 0.1% BSA. Assay accuracy was controlled by endogenous quality controls (Table 1). Internal standards working solutions (ISWS) were prepared in methanol with final concentrations at mid-range of the calibration curve. Total brain tissue was used to prepare the EndoQCs.

Blocks from all the main monkey brain areas were cut, frozen at −80 °C and then sliced at 50 µm intervals with a cryostat (temperature set at −20 °C). Mortar and pestle were previously cleaned with 14% nitric acid in ultrapure water. Then, they were thoroughly rinsed with ultrapure water and finally pure methanol in order to eliminate surface impurities. Slices were pooled in the mortar and crushed with a pestle, both cooled on dry ice, until tissue was in a powder state. The homogenous powder was then

divided in 2 mL microtubes (kept cooled on dry ice) containing 4 stainless steel metal beads (2.38 mm), both obtained from VWR (QC, Canada), in order to weigh as close as possible 0.2 g of tissue per tube. The EndoQC was calculated from at least six separate replicates from which the coefficient of variation was less than ±15%. Quantification accuracies of endogenous QCs were normalized with the exact mass of the tissue in each tube (Fig. 1).

2.4. Samples, standard curves and QC preparations

0.1 mL of ISWS was spiked in tubes containing tissue samples or EndoQCs and kept on dry ice, while 0.1 mL of methanol was added in tissue blank tubes. 0.3 mL of methanol was then added into each tube. The content was then homogenized with a Bead Ruptor (Omni International, GA, USA) at high speed for 90 s and then placed on melting ice. 1 mL of methanol was then added into tubes which were homogenized again before centrifugation (7628×*g*) at 4 °C for 5 min. Supernatants were transferred in borosilicate tubes. Samples and EndoQCs were rehomogenized with 1.2 mL of methanol, centrifuged and the supernatants were pooled in individual borosilicate tubes. To precipitate protein, supernatants were then flash-cooled in an acetone dry-ice bath for 3 min and tubes were then centrifuged (3434×*g*) at 4 °C for 5 min. Supernatants were then decanted into clean borosilicate tubes. The pellets obtained were washed with 1 mL of methanol, vortexed, snap-cooled, centrifuged and decanted into their respective tubes. The total methanol volume in each tube was evaporated under a constant flow of gas nitrogen before being resuspended in 0.8 mL of 1% formic acid in methanol. Tubes were then put in a sonic bath for 5 min to optimize the resuspension. The suspension was eluted through a solid phase extraction phospholipid removal plate (Phree, Phenomenex, CA, USA) with the help of a Waters Positive Pressure-96 Processor (MA, USA) under gas nitrogen at 3 psi. Each well was then washed with 0.5 mL of 1% formic acid in methanol. The content of each well was evaporated under gas nitrogen (SPE Dry-96 Dual, Biotage, NC, USA) and then resuspended in 0.8 mL of 1-chlorobutane (Fig. 1).

2.5. The calibration curve was prepared in duplicate

0.2 mL of STD curve and QC solution (Table 1) were placed in a borosilicate tube in which 0.1 mL of ISWS was spiked (except blank) and mixed with 3 mL of 1-chlorobutane before centrifugation (2670×*g*) at 4 °C for 5 min. Each tube was then snap-frozen in an acetone dry-ice bath for 3 min and the supernatants were decanted in clean borosilicate tubes. Samples, EndoQCs, STD curves and QCs which have been resuspended in 1-chlorobutane were passed through conditioned (2.5 mL of ethyl acetate, then hexanes, then 1-chlorobutane) silica columns (Strata SI-1 silica, 55 µm, 70A, Phenomenex, CA, USA). After transferring the resuspended tissue samples and EndoQCs onto the silica columns, the wells were washed with another 0.8 mL of 1-chlorobutane and then transferred onto respective silica columns. Columns were washed with 4 mL of hexanes and their contents were eluted with 4 mL of ethyl acetate/hexanes (1:1; v/v) in clean borosilicate tubes and finally evaporated under a constant flow of nitrogen. A different percentage of ethyl acetate in the elution solution could affect the S/N ratio of LLOQs and the sensitivity of detection of endogenous steroids, especially DHT, E_1 and E_2. Residues on the side of the tubes were concentrated towards the bottom with 0.4 mL of ethyl acetate/hexanes (1:1; v/v) which was then evaporated (Fig. 1).

Specific derivatization of estrogens was performed with 50 µL of a dansyl chloride solution in acetone (0.85 mg/mL) and 50 µL of sodium bicarbonate 0.2 M at 60 °C for 10 min before being snap-frozen in a dry-ice acetone bath. Once thawed, 100 µL of diluent (50% MeOH, 10 mM ammonium formate and 0.2% formic acid in ultrapure water) was added to each tube which was then thoroughly vortexed and centrifuged. The volume of each tube was transferred onto an Impact filter plate (Phenomenex, CA, USA) which is set on top of an autosampling 96-deep square well plate (Canadian Life Science, ON, Canada) and centrifuged at 3434×*g* (4 °C for 5 min) in order to eliminate fine particles that may block the column. 60 µl of the eluate in every well was distributed in a new 96-deep well plate (Fig. 1). Samples may be reconstituted directly in diluent without derivatization if only DHEA, 4-dione, 5-diol and DHT are to be quantified.

2.6. LC–MS/MS

30 µL was injected for the quantification of unconjugated estrogen-related compounds, while another 30 µL was injected to quantify unconjugated androgen-related steroids on a UPLC (LC-30AD system; Shimadzu; ON, Canada)-MS/MS (Qtrap 6500 system; AB Sciex, ON, Canada) with a MRM (Multiple Reaction Monitoring) mode (Table 2). The analytical column used was an Agilent Poroshell 120 EC-C18 (3.0 × 50 mm; 2.7 µm) with an in-line filter (Waters, MA, USA) for androgen-related steroid analytes. A poroshell 120 SB-C18 (2.1 mm × 30 mm, 2.7 µm) (Agilent, CA, USA) with an in-line filter (Waters MA, USA) followed by a SB-C18 rapid resolution HD (2.1 mm × 50 mm, 1.8 µm) (Agilent) was used for estrogen-related steroid analytes. 0.2%

Table 2
MRM transitions and system specifications for the measurement of all seven analytes and their internal standards.

Analyte ID	Q1 Mass	Q3 Mass	Dwell Time	DP-Volts (start/stop)	CE-Volts (start/stop)
E_2	506.2	171.1	150	100/100	45/45
E_2-d4	510.2	171.1	75	100/100	45/45
E_1	504.2	171.1	100	80/80	51/51
E_1-d4	508.2	171.1	75	80/80	51/51
DHT	291.2	255.2	200	40/40	21/21
DHT-d3	294.1	258.2	75	40/40	21/21
DHEA	271.2	213.2	100	120/120	30/30
DHEA-d5	276.2	218.2	100	120/120	30/30
Testo	289.2	97.2	75	100/100	33/33
4-dione	287.2	97.2	75	80/80	33/33
4-dione-d7 and Testo-d5	294.2	100.2	50	100/100	33/33
5-diol-d3	276.2	161.2	75	50/50	29.5/29.5
5-diol	273.2	159.2	150	50/50	29.5/29.5

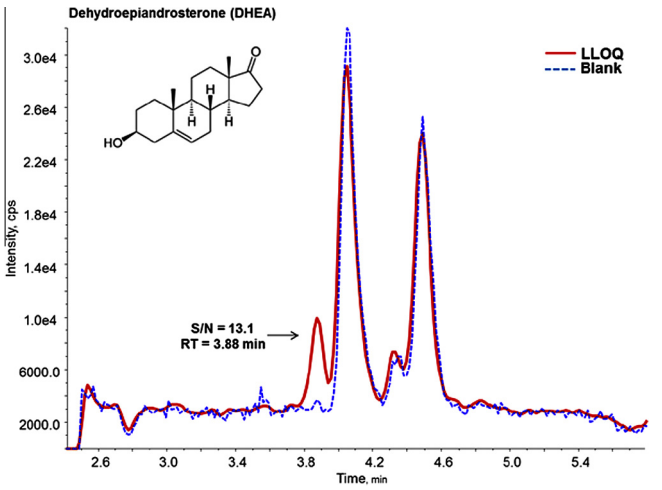

Fig. 2A. Representative chromatograms of the LLOQs over blank for DHEA along with respective retention times and signal-to-noise ratios for each analyte.

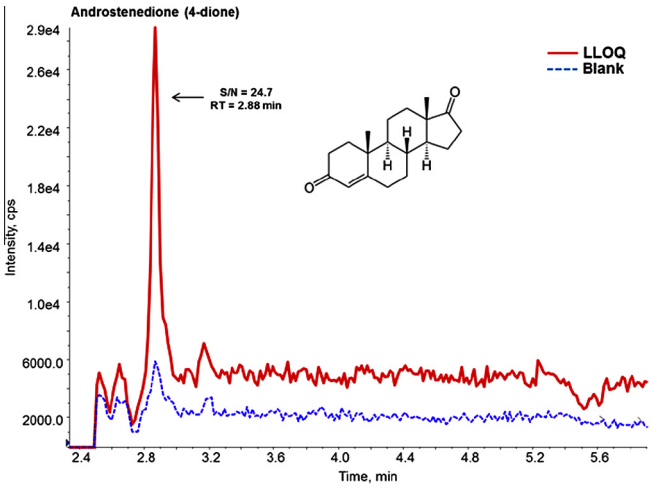

Fig. 2B. Representative chromatograms of the LLOQs over blank for 4-dione along with respective retention times and signal-to-noise ratios for each analyte.

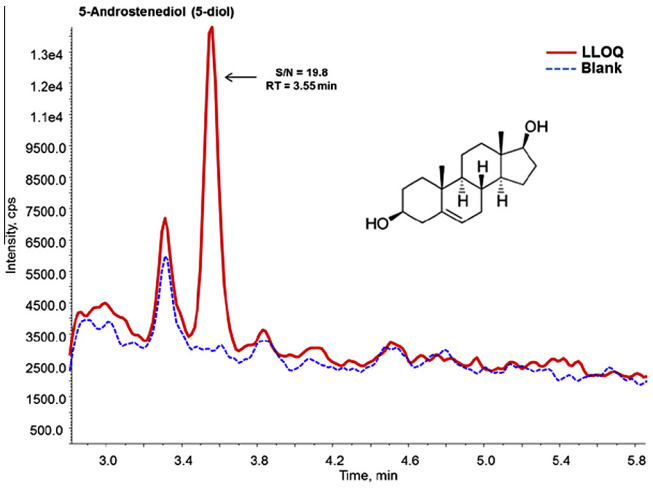

Fig. 2C. Representative chromatograms of the LLOQs over blank for 5-diol along with respective retention times and signal-to-noise ratios for each analyte.

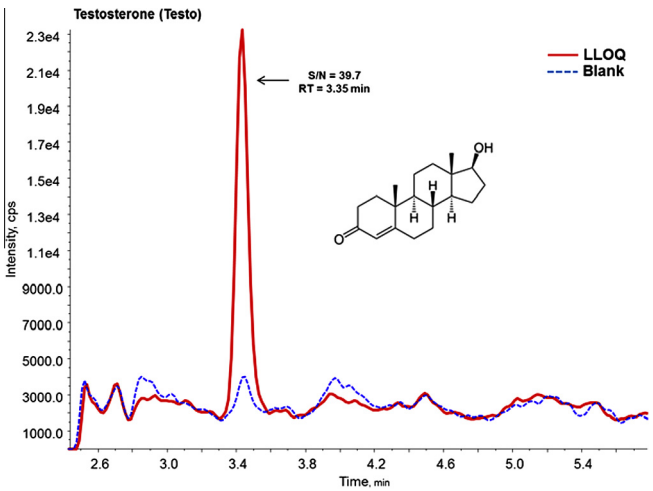

Fig. 2D. Representative chromatograms of the LLOQs over blank for Testo along with respective retention times and signal-to-noise ratios for each analyte.

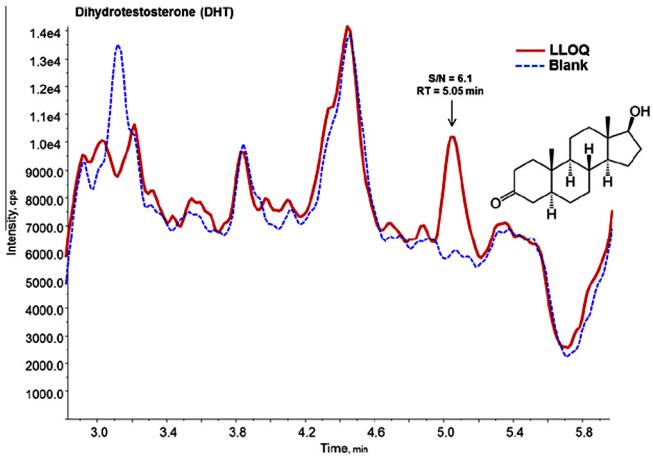

Fig. 2E. Representative chromatograms of the LLOQs over blank for DHT along with respective retention times and signal-to-noise ratios for each analyte.

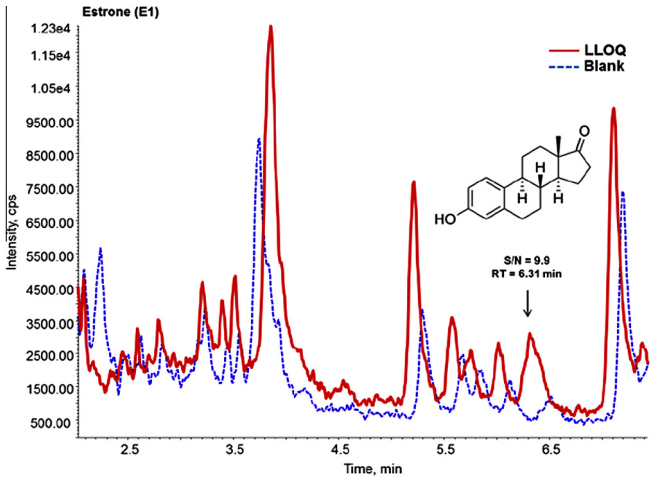

Fig. 2F. Representative chromatograms of the LLOQs over blank for E_1 along with respective retention times and signal-to-noise ratios for each analyte.

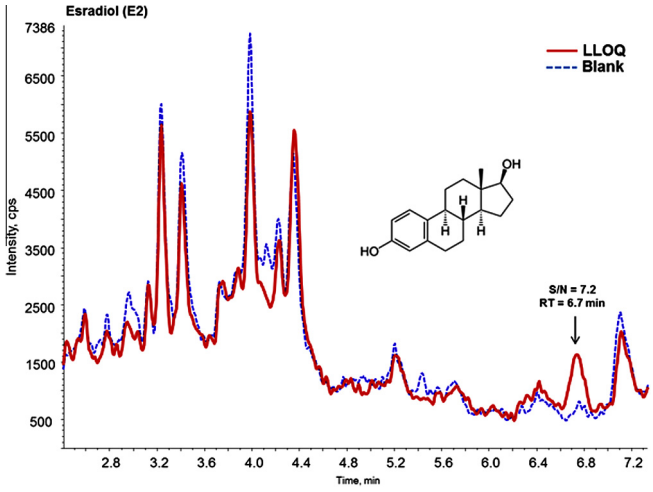

Fig. 2G. Representative chromatograms of the LLOQs over blank for E_2 along with respective retention times and signal-to-noise ratios for each analyte.

formic acid in water and MeOH were used as weak and strong mobile phases, respectively, for estrogens and androgens (Fig. 1).

3. Results

3.1. Selectivity, sensitivity and specificity

The sample preparation combines a liquid extraction of analytes from the tissue matrix followed by solid-phase purification and finally a specific derivatization of the E_1 and E_2 phenol group, thus increasing the proton affinity of estrogen-related steroids and the sensitivity by LC–ESI-MS/MS for their dominant fragment product ion $m/z = 171$ [51,52]. Unconjugated androgen-related analytes do not require derivatization because of their natural high ionization potential and higher serum concentrations. Thus, a simple sample preparation is feasible to obtain LLOQs of 1 pg/mL, 4 pg/mL, 10 pg/mL, 50 pg/mL, 200 pg/mL, 12 pg/mL and 250 pg/mL for E_2, E_1, DHT, Testo, 5-diol, 4-dione and DHEA, respectively (Table 1).

The chromatograms of the analytes over blank as well as the signal/noise (S/N) ratio of the LLOQs observed for the seven investigated compounds whereas the E_1, E_2 and DHT show the lowest but significant responses over blank (Fig. 2). The blank preparation was identical to that of the LLOQs, except that it includes only internal standards with no spiked analytes. The blank shows no detectable interference for the analytes coming from the matrix used for the QCs and STD curves. Furthermore, the blank shows no significant signal for all seven analytes indicating that there is no contribution towards the analytes' quantification by their respective internal standard. It should be noted that reagent alcohol and ethanol were tried as extraction solvents, with higher S/N ratio of LLOQs, thus resulting in higher background levels (data not shown).

The tissue blanks (without internal standards) show no significant signal at the mass transitions shown in Table 2 at the retention times specific for all 7 internal standards (Fig. 3). Indeed, using this method, no matrix component significantly alters the quantification of all seven analytes. Moreover, internal standards for 4-dione and Testo share the same MRM transition but are discriminated according to their respective retention times. The LC separation combined with the tandem MS system permits to differentiate isobaric DHEA and Testo by their retention times and MRM transitions. On top of the MRM analysis which permits to specifically measure the analytes of interest by tandem mass spectrometry, the UPLC column provides a higher resolution of

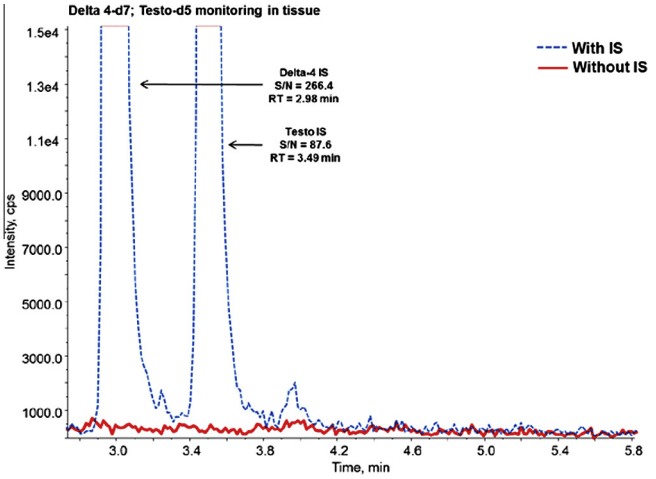

Fig. 3B. Representative chromatograms of tissue blanks showing no matrix contribution at the expected retention times and mass transitions for 4-dione-d7; Testo-d5.

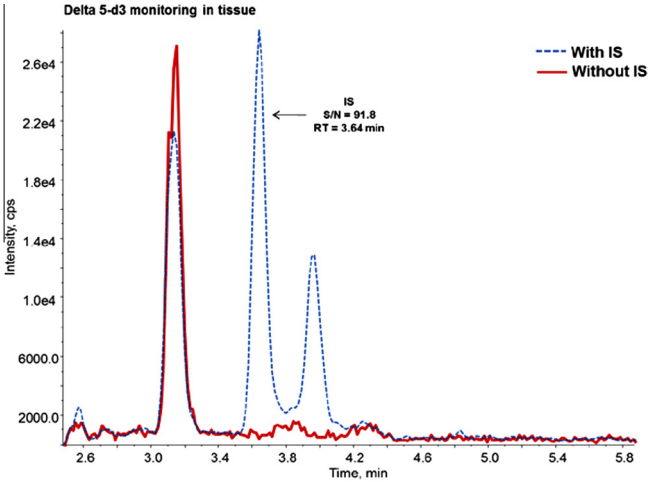

Fig. 3C. Representative chromatograms of tissue blanks showing no matrix contribution at the expected retention times and mass transitions for 5-diol-d3.

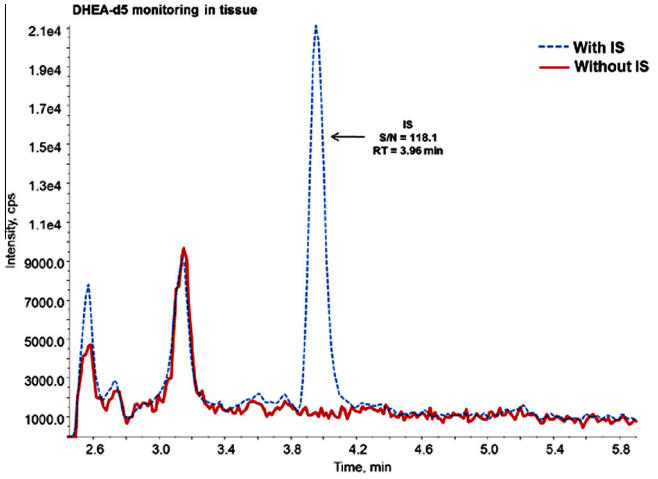

Fig. 3A. Representative chromatograms of tissue blanks showing no matrix contribution at the expected retention times and mass transitions for DHEA-d5.

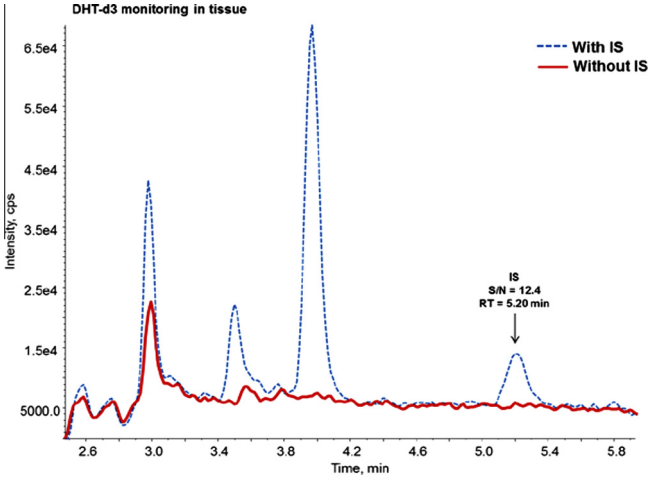

Fig. 3D. Representative chromatograms of tissue blanks showing no matrix contribution at the expected retention times and mass transitions for DHT-d3.

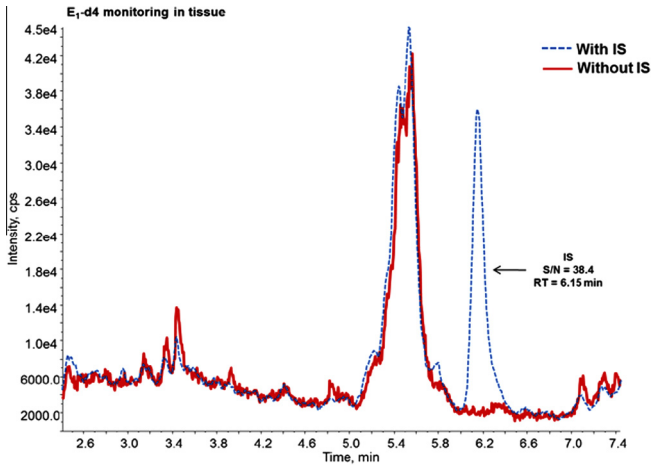

Fig. 3E. Representative chromatograms of tissue blanks showing no matrix contribution at the expected retention times and mass transitions for E_1-d4.

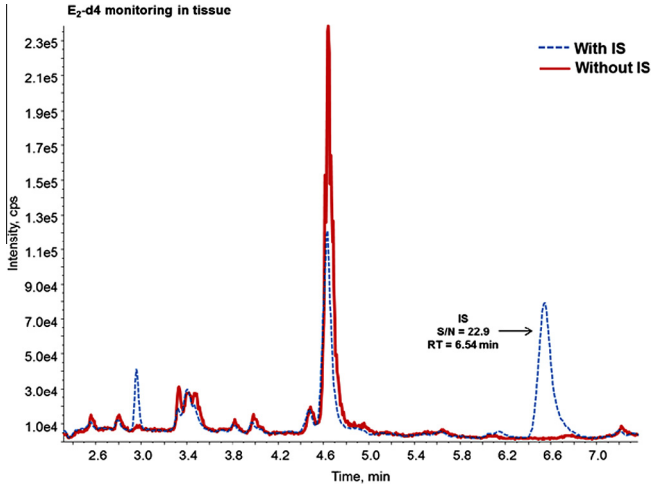

Fig. 3F. Representative chromatograms of tissue blanks showing no matrix contribution at the expected retention times and mass transitions for E_2-d4.

analytes compared to conventional HPLC and adds to the sensitivity limit due to its well defined peaks for all seven compounds utilizing the UPLC-6500 Sciex Qtrap system.

As shown in Fig. 4, well defined peaks in total brain tissue extracts are achieved for endogenous DHEA, 4-dione, 5-diol, Testo, DHT, E_1 and E_2 with S/N ratios significantly exceeding the LLOQ (Fig. 4). These steroids could, however, possibly become more abundant in specific regions of the brain. Endogenous levels of brain steroids are shown only as chromatograms since total brain homogenate was used.

3.2. Calibration curves

The calibration curve linearity and its QC's accuracies in 0.1% BSA/water were evaluated for all seven compounds. Calibration curves in stripped serum, 0.1% BSA/water and analyte-spiked brain tissue homogenate all showed a linearity regression correlation $\geqslant$0.99 with QC accuracies of 100 ± 15% for all QC levels (data not shown). Therefore, BSA in ultrapure water was selected as a matrix for the calibration curves and QCs since it also offers consistently low background levels and is free from interferences, resulting in a reliable and accurate quantification of EndoQCs in brain tissue matrix. It should be noted that when a higher percentage of BSA

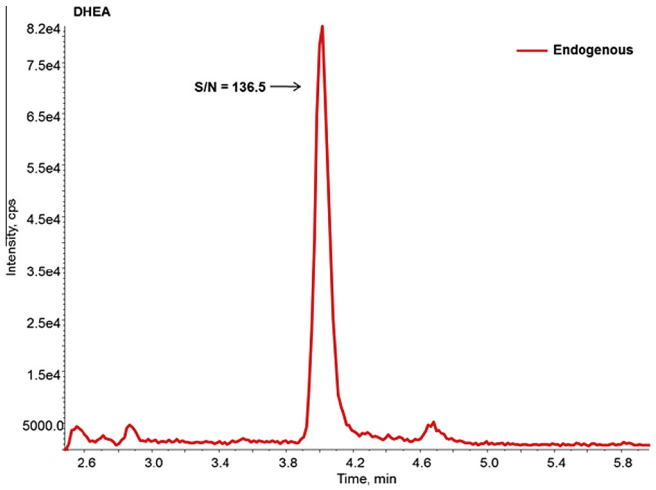

Fig. 4A. Detection of endogenous levels of DHEA in intact male monkey brain tissue homogenate.

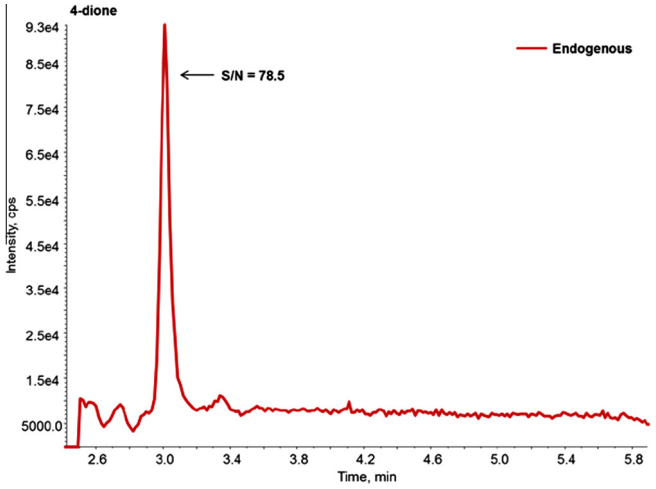

Fig. 4B. Detection of endogenous levels of 4-dione in intact male monkey brain tissue homogenate.

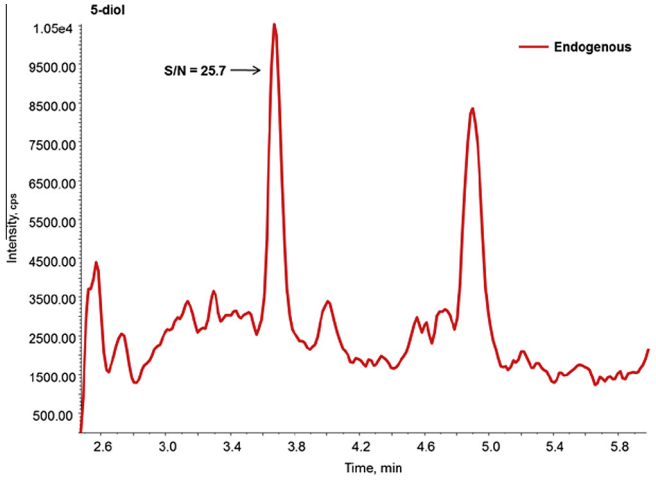

Fig. 4C. Detection of endogenous levels of 5-diol in intact male monkey brain tissue homogenate.

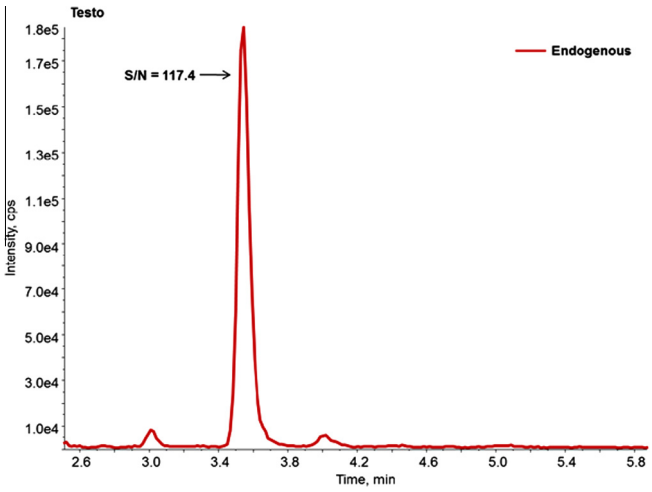

Fig. 4D. Detection of endogenous levels of Testo in intact male monkey brain tissue homogenate.

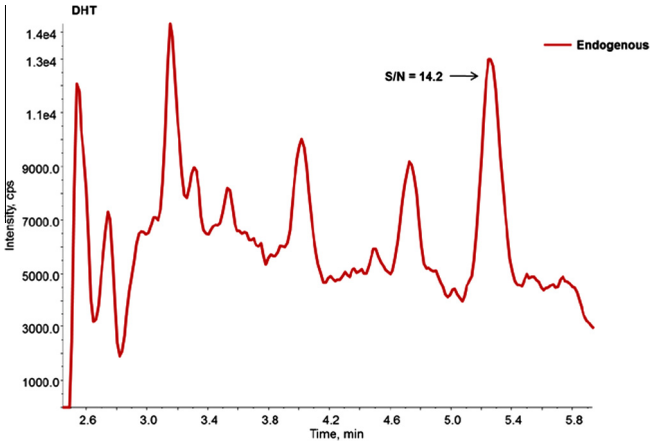

Fig. 4E. Detection of endogenous levels of DHT in intact male monkey brain tissue homogenate.

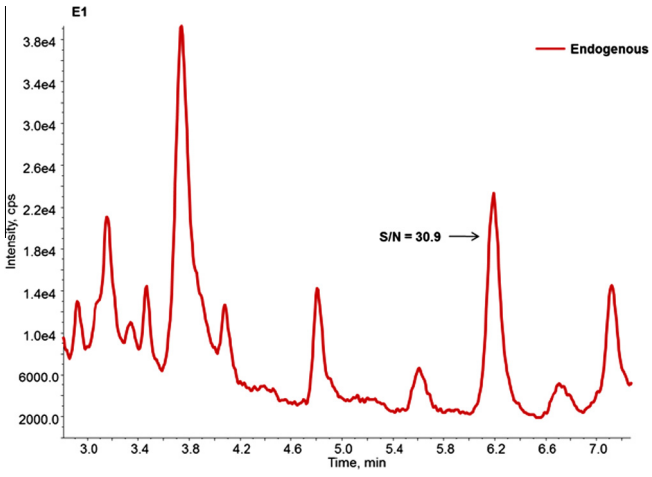

Fig. 4F. Detection of endogenous levels of E_1 in intact male monkey brain tissue homogenate.

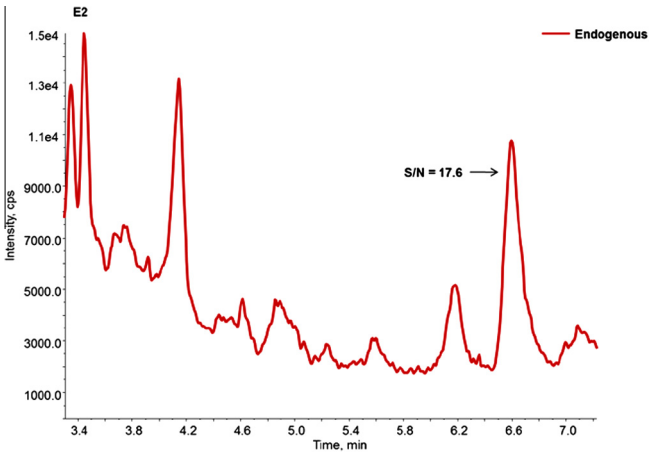

Fig. 4G. Detection of endogenous levels of E_2 in intact male monkey brain tissue homogenate.

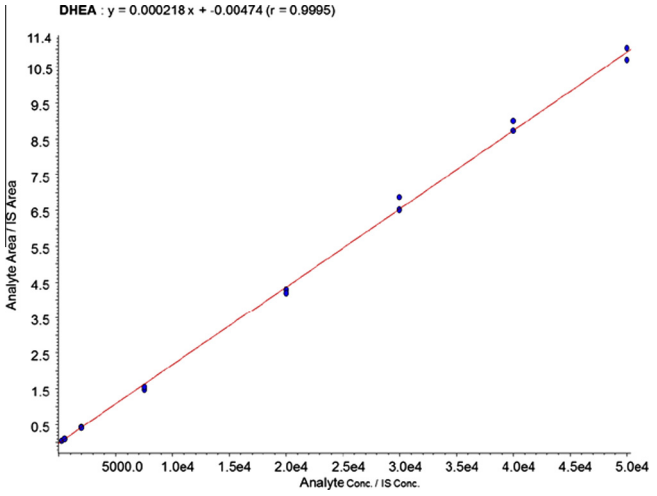

Fig. 5A. Linearity of the calibration curves and their correlation factor in ultrapure water containing 1% BSA for DHEA.

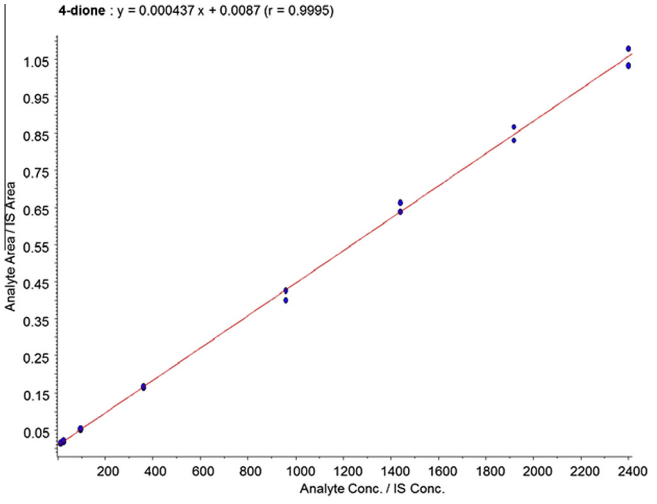

Fig. 5B. Linearity of the calibration curves and their correlation factor in ultrapure water containing 1% BSA for 4-dione.

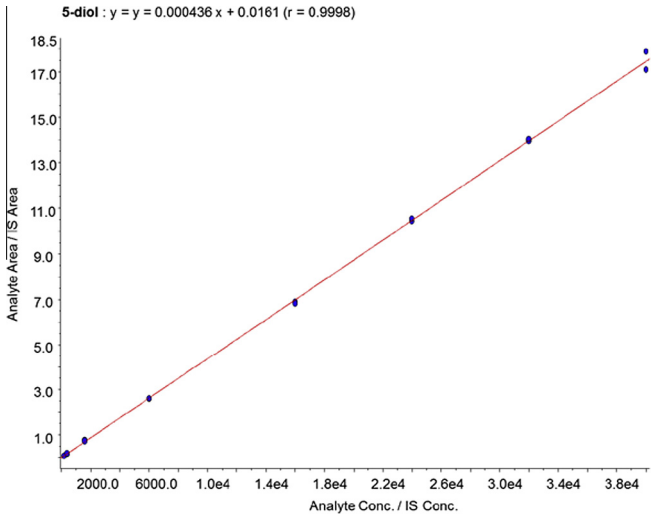

Fig. 5C. Linearity of the calibration curves and their correlation factor in ultrapure water containing 1% BSA for 5-diol.

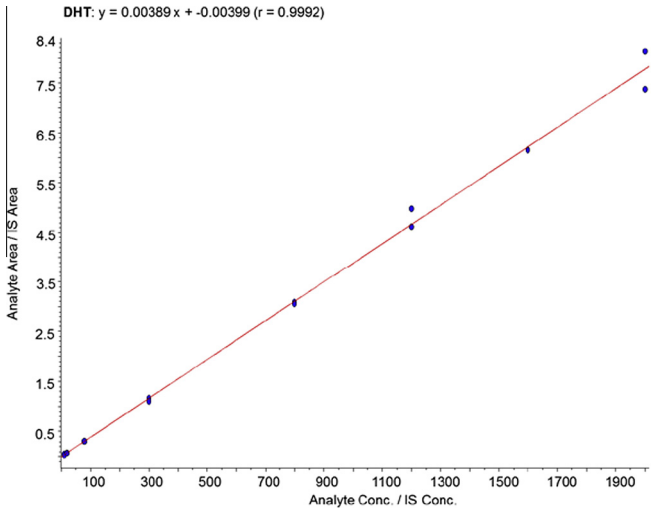

Fig. 5D. Linearity of the calibration curves and their correlation factor in ultrapure water containing 1% BSA for DHT.

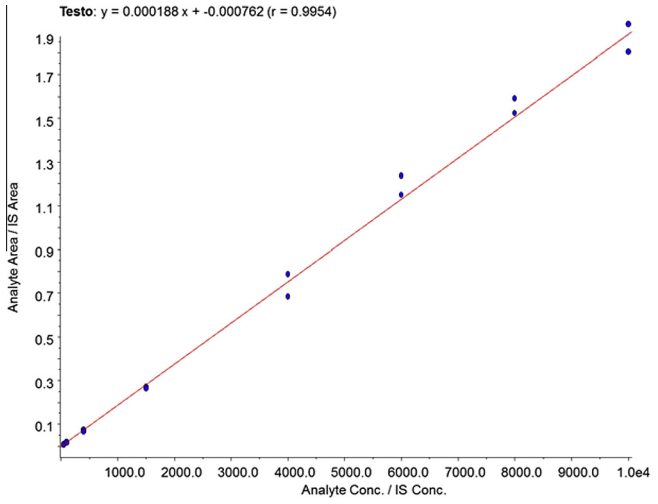

Fig. 5E. Linearity of the calibration curves and their correlation factor in ultrapure water containing 1% BSA for Testo.

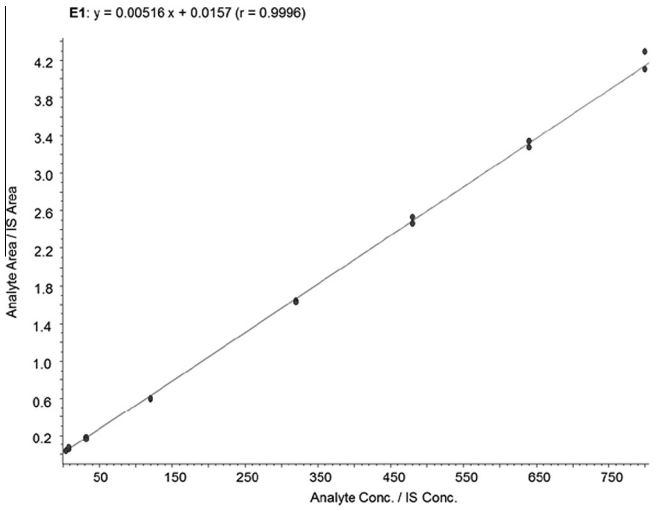

Fig. 5F. Linearity of the calibration curves and their correlation factor in ultrapure water containing 1% BSA for E_1.

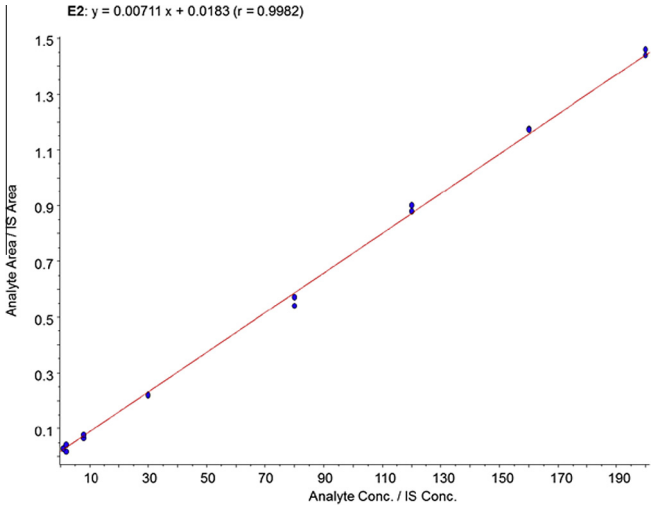

Fig. 5G. Linearity of the calibration curves and their correlation factor in ultrapure water containing 1% BSA for E_2.

was used, e.i. 1%, some impurities were detected in the calibration curve's blank, thus interfering with the quantification. Shown in Fig. 5 is the linearity of the calibration curves for all seven compounds with an $R > 0.99$. Table 3 depicts the values and accuracies at 100 ± 15% for all eight points of the seven analyte's standard curves. The weighting factor utilized was $1/\times$.

3.3. Quality controls and accuracies

QC precisions and accuracies for the seven compounds are presented in Table 4. The coefficient of variation for all levels of QCs is lower than the ±15% in the BSA/water matrix recommended by the guidelines of the regulatory agencies. EndoQC accuracies were calculated in three different assays where pulverized brain material was thoroughly mixed until homogenous and then aliquoted at 0.2 g per tube (Fig. 1). Prior to extraction, tubes were spiked (or not for tissue blanks) with the internal standard working solution and different levels of unconjugated steroids for EndoLowQC, EndoMedQC and EndoHighQC. Only internal standards were spiked for the EndoQCs which were used to determine the endogenous

Table 3
Measurement and accuracy of the calibrants.

	Expected	Mean	%CV	Accuracy
DHEA				
StdB	250	268.8	0.8	107.5
StdC	500	505.2	6.5	101.0
StdD	2000	1957.1	3.4	97.8
StdE	7500	6970.4	3.4	92.9
StdF	20,000	19,422.9	1.6	97.1
StdG	30,000	30,710.4	3.7	102.3
StdH	40,000	40,626.5	2.2	101.5
StdI	50,000	49,788.2	2.1	99.5
4-dione				
StdB	12	11.5	32.6	96.0
StdC	24	24.1	19.5	100.4
StdD	96	100.9	3.8	105.1
StdE	360	359.3	0.4	99.8
StdF	960	924.1	4.5	96.2
StdG	1440	1469.3	2.6	102.0
StdH	1920	1924.2	3.0	100.2
StdI	2400	2398.3	3.1	99.9
5-diol				
StdB	200	188.9	1.4	94.4
StdC	400	407.6	4.6	101.9
StdD	1600	1685.7	3.5	105.3
StdE	6000	5960.2	0.3	99.3
StdF	16,000	15,696.8	0.7	98.1
StdG	24,000	24,077.1	0.7	100.3
StdH	32,000	32,063.1	0.4	100.1
StdI	40,000	40,120.1	3.2	100.3
Testo				
StdB	50	51.8	14.3	103.6
StdC	100	102.9	4.2	102.9
StdD	400	384.2	7.2	96.0
StdE	1500	1432.4	1.5	95.4
StdF	4000	3783.4	15.3	94.5
StdG	6000	6338.3	5.1	105.6
StdH	8000	8795.1	2.8	109.9
StdI	10,000	9161.6	7.3	91.6
DHT				
StdB	10	10.2	28.7	102.6
StdC	20	19.7	3.2	98.7
StdD	80	79.7	1.4	99.6
StdE	300	294.7	3.6	98.2
StdF	800	792.6	0.6	99.0
StdG	1200	1235.2	5.4	102.9
StdH	1600	1583.0	0.2	98.9
StdI	2000	1994.5	6.8	99.7
E_1				
StdB	4	3.7	10.2	93.9
StdC	8	9.3	23.9	117.1
StdD	32	30.0	8.2	93.7
StdE	120	114.2	0.2	95.2
StdF	320	314.3	0.9	98.2
StdG	480	481.6	1.9	100.3
StdH	640	638.6	1.5	99.7
StdI	800	812.0	3.2	101.5
E_2				
StdB	1	0.8	3.2	89.4
StdC	2	2.0	35.3	102.1
StdD	8	8.9	12.1	111.9
StdE	30	29.6	0.6	98.7
StdF	80	76.0	4.0	95.0
StdG	120	122.0	1.7	101.7
StdH	160	161.5	0.1	100.9
StdI	200	199.8	0.9	99.9

levels of steroids per gram of tissue. Taking into account the known endogenous concentration of steroids per gram of pulverized brain homogenate and the spiked amount of analytes, accuracies for all three EndoQC levels were calculated. Briefly, EndoQCs are initially obtained as pg/mL because of the calibration curve which is also in pg/mL. The EndoQC concentration is then converted per gram of tissue in order to compare the same mass (0.2 g: same volume

Table 4
Measurement and accuracy of the calibrants' quality controls.

	Expected	Mean	%CV	Accuracy
DHEA				
LLOQ	250	210.8	2.1	84.3
LOWQC	750	779.1	3.6	103.8
MEDQC	25,000	25,065.8	5.4	100.2
HIGHQC	37,500	42,391.5	1.9	113.0
4-dione				
LLOQ	12	10.6	41.1	88.6
LOWQC	36	26.7	16.3	74.1
MEDQC	1200	1170.7	0.27	97.5
HIGHQC	1800	2023.5	1.7	112.4
5-diol				
LLOQ	200	166.1	2.8	82.9
LOWQC	600	617.7	2.8	102.9
MEDQC	20,000	19,395.2	1.8	96.9
HIGHQC	30,000	33,182.3	1.1	110.5
Testo				
LLOQ	50	47.2	2.2	94.4
LOWQC	150	137.6	2.1	91.7
MEDQC	5000	4821.2	1.7	96.3
HIGHQC	7500	8227.3	1.1	109.6
DHT				
LLOQ	10	10.2	28.7	102.6
LOWQC	30	32.1	9.5	106.7
MEDQC	1000	971.8	5.2	97.1
HIGHQC	1500	1630.2	5.1	108.6
E_1				
LLOQ	4	3.4	20.3	85.7
LOWQC	12	12.3	6.9	103.1
MEDQC	400	396.8	0.6	99.2
HIGHQC	600	669.6	0.6	111.5
E_2				
LLOQ	1	0.9	3.2	89.4
LOWQC	3	2.1	74.1	79.1
MEDQC	100	95.1	9.5	95.1
HIGHQC	150	169.3	2.3	112.8

extracted for each point of the calibration curve and QC). Higher tissue weights result in dirtier reconstituted samples and thus suppress signal at the MS. On the other hand, some endogenous steroids are undetected when using too small sample sizes. After determining the EndoQC level, it is then possible to calculate the accuracy of the spiked levels (EndoLowQC, EndoMedQC and EndoHighQC). Table 5 shows the EndoQCs mean accuracies, standard deviation and coefficient of variation for all seven analytes.

4. Discussion

The extraction methods and derivatization procedures for tissue steroid assays often use the prostate, skin and breast [24,53–55], as well as the adrenal gland, testis, liver, ovary and uterus, as examples [56,57]. With the availability of more sensitive mass spectrometers and ultra-performant liquid chromatography (UPLC), LC–MS/MS has become an efficient technique for the measurement of steroids with low limits of quantitation (LLOQ) with values down to the 5 pg/mL level for Testo and 40 pg/mL for 4-dione without derivatization [58]. For compounds such as E_1 and E_2, which do not ionize efficiently, a specific derivatization of these compounds permits a LLOQ detection down to the lowest pg/mL level [59] using dansyl chloride [29,52,59–61].

Interfering peaks in biological matrices which co-elute with the analytes of interest can cause ionization suppression, thus reducing the detection sensitivity [62]. This is especially true with brain tissue samples due to their high lipid content which is notorious for signal suppression at the mass spectrometry level, a complexity to be corrected by adequate sample preparation. In the present study, freshly perfused and snap-frozen monkey brain tissue was

Table 5
Measurement and accuracy of endogenous quality controls.

	SD	%CV	Accuracy
DHEA			
Low EndoQC	508.8	4.5	102.5
Med EndoQC	1058.9	3.1	104.1
High EndoQC	1489.8	3.4	108.0
	SD	Mean	Accuracy
4-dione			
Low EndoQC	6.8	3.1	114.1
Med EndoQC	884.8	41.5	108.7
High EndoQC	270.6	12.3	113.7
5-diol			
Low EndoQC	35.1	3.4	112.9
Med EndoQC	403.2	2.2	110.1
High EndoQC	3579.6	12.6	99.7
	SD	%CV	Accuracy
DHT			
Low EndoQC	11.8	8.6	98.5
Med EndoQC	69.3	7.2	107.5
High EndoQC	57.7	3.8	112.4
Testo			
Low EndoQC	17.7	3.7	95.5
Med EndoQC	596.5	12.9	96.1
High EndoQC	818.9	11.4	101.7
E_1			
Low EndoQC	4.5	9.6	107.7
Med EndoQC	22.1	6.6	111.6
High EndoQC	37.6	7.3	107.1
E_2			
Low EndoQC	0.7	8.4	100.1
Med EndoQC	14.9	17.7	97.7
High EndoQC	9.1	8.3	106.3

used to avoid contamination by the blood and steroid metabolism, respectively. This approach was necessary to develop a method allowing a sensitive, specific and accurate assay of brain steroids.

In the present report, a sensitive and robust UPLC-MS/MS method has been developed for the accurate quantification of the seven steroids in brain tissue. To our knowledge, this is the first described method for the measurement of seven unconjugated steroids simultaneously in the brain or any other tissue matrix by mass spectrometry. The UPLC column, with more separation power, improves both the specificity and the sensitivity with a better signal to noise ratio at the LC–MS/MS level, thus permitting a low limit of quantitation with LLOQs of 1 pg/mL and 10 pg/mL for E_2 and DHT, respectively. Such sensitivity and specificity are crucial to assess the possible contribution of various neurosteroids in neurocognitive functions and impairment, a role which may be brain-region specific. Moreover, a low limit of detection is needed to respond to the challenge of variable background which may vary according to the brain region analyzed. The matrix components present in the cerebral cortex (essentially composed of gray matter) and in the underlaying white matter compartment are likely to be quite different, thus complicating the steroid assays. While 5-diol and E_2 were present at relatively low levels in whole cynomolgus brain homogenates, these steroids may well be present at higher levels in specific brain areas.

The method presented herein could potentially be applied to quantify these seven steroids in other less lipemic tissues. Indeed, our group has measured these steroids in the monkey prostate, seminal vesicles, muscle (preliminary data) and vagina [63,64]. This technology could thus open the way for studies to better understanding the role of DHEA and its unconjugated metabolites in the brain and other tissues. More specifically, the use of this technology should help explain the role of neurosteroids made locally from DHEA in brain physiology and neurologic diseases, especially cognitive impairment and AD.

Neurodegenerative diseases, in particular AD, are believed to arise from a multifactorial process which ultimately leads to a decline in neural plasticity, neuroregenerative transformation, and development of amyloid-beta (Aβ) plaques and neurofibrillary tangles [65], particularly in the hippocampus and cortex [66]. Since it is now believed that brain steroids could have a role in the development and progression of AD and other neurocognitive impairments, an accurate quantification of unconjugated steroids in the brain is very important in order to understand explain how these compounds could participate in the onset/progression of neurologic diseases. Complex sample preparation is one of the main hurdles to the accurate quantification of brain steroids.

Conflict of interest

The authors declare no conflict of interest.

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References

[1] Labrie F, Martel C, Balser J. Wide distribution of the serum dehydroepiandrosterone and sex steroid levels in postmenopausal women: role of the ovary? Menopause 2011;18:30–43.
[2] Labrie F, Bélanger A, Cusan L, Gomez JL, Candas B. Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. J. Clin. Endocrinol. Metab. 1997;82:2396–402.
[3] Labrie F, Labrie C. DHEA and intracrinology at menopause, a positive choice for evolution of the human species. Climacteric 2013;16:205–13.
[4] Beaulieu M, Lévesque É, Hum DW, Bélanger A. Isolation and characterization of a human orphan UDP-glucuronosyltransferase, UGT2B11. Biochem. Biophys. Res. Commun. 1998;248:44–50.
[5] Sakamoto H, Ukena K, Tsutsui K. Effects of progesterone synthesized de novo in the developing Purkinje cell on its dendritic growth and synaptogenesis. J. Neurosci. 2001;21:6221–32.
[6] Melcangi RC, Panzica GC. Neuroactive steroids: old players in a new game. Neuroscience 2006;138:733–9.
[7] Sorrells SF, Caso JR, Munhoz CD, Sapolsky RM. The stressed CNS: when glucocorticoids aggravate inflammation. Neuron 2009;64:33–9.
[8] Honda SI, Wakatsuki T, Harada N. Behavioral analysis of genetically modified mice indicates essential roles of neurosteroidal estrogen. Front. Endocrinol. 2011;2:1–8.
[9] Corpechot C, Robel P, Axelson M, Sjovall J, Baulieu EE. Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. Proc. Natl. Acad. Sci. U.S.A. 1981;78:4704–7.
[10] Compagnone NA, Mellon SH. Neurosteroids: biosynthesis and function of these novel neuromodulators. Front. Neuroendocrinol. 2000;21:1–56.
[11] Mellon SH, Griffin LD. Neurosteroids: biochemistry and clinical significance. Trends Endocrinol. Metab. 2002;13:35–43.
[12] Schmidt KL, Pradhan DS, Shah AH, Charlier TD, Chin EH, Soma KK. Neurosteroids, immunosteroids, and the Balkanization of endocrinology. Gen. Comp. Endocrinol. 2008;157:266–74.
[13] Do Rego JL, Seong JY, Burel D, Leprince J, Luu-The V, Tsutsui K, et al. Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides. Front. Neuroendocrinol. 2009;30:259–301.
[14] Taves MD, Gomez-Sanchez CE, Soma KK. Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. Am. J. Physiol. Endocrinol. Metab. 2011;301:E11–24.
[15] Lacroix C, Fiet J, Benais JP, Gueux B, Bonete R, Villette JM, et al. Simultaneous radioimmunoassay of progesterone, androst-4-enedione, pregnenolone, dehydroepiandrosterone and 17-hydroxyprogesterone in specific regions of human brain. J. Steroid. Biochem. 1987;28:317–25.
[16] Liere P, Pianos A, Eychenne B, Cambourg A, Liu S, Griffiths W, et al. Novel lipoidal derivatives of pregnenolone and dehydroepiandrosterone and absence of their sulfated counterparts in rodent brain. J. Lipid Res. 2004;45:2287–302.
[17] Lapchak PA, Araujo DM. Preclinical development of neurosteroids as neuroprotective agents for the treatment of neurodegenerative diseases. Int. Rev. Neurobiol. 2001;46:379–97.
[18] Azcoitia I, Leonelli E, Magnaghi V, Veiga S, Garcia-Segura LM, Melcangi RC. Progesterone and its derivatives dihydroprogesterone and tetrahydroprogesterone reduce myelin fiber morphological abnormalities

and myelin fiber loss in the sciatic nerve of aged rats. Neurobiol. Aging 2003;24:853–60.
[19] McCullough LD, Hurn PD. Estrogen and ischemic neuroprotection: an integrated view. Trends Endocrinol. Metab. 2003;14:228–35.
[20] Ciriza I, Azcoitia I, Garcia-Segura LM. Reduced progesterone metabolites protect rat hippocampal neurones from kainic acid excitotoxicity in vivo. J. Neuroendocrinol. 2004;16:58–63.
[21] Yan G, Zhang G, Fang X, Zhang Y, Li C, Ling F, et al. Genome sequencing and comparison of two nonhuman primate animal models, the cynomolgus and Chinese rhesus macaques. Nat. Biotechnol. 2011;29:1019–23.
[22] Luu-The V, Labrie F. The intracrine sex steroid biosynthesis pathways. In: Martini L, Chrousos GP, Labrie F, Pacak K, Pfaff De, editors. Neuroendocrinology, Pathological Situations and Diseases, Progress in Brain Research. Elsevier; 2010. p. 177–92.
[23] Yamashita K, Okuyama M, Watanabe Y, Honma S, Kobayashi S, Numazawa M. Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography–electrospray ionization tandem mass spectrometry. Steroids 2007;72:819–27.
[24] Yamashita K, Miyashiro Y, Maekubo H, Okuyama M, Honma S, Takahashi M, et al. Development of highly sensitive quantification method for testosterone and dihydrotestosterone in human serum and prostate tissue by liquid chro matography–electrospray ionization tandem mass spectrometry. Steroids 2009;74:920–6.
[25] Matsui F, Koh E, Yamamoto K, Sugimoto K, Sin HS, Maeda Y, et al. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for simultaneous measurement of salivary testosterone and cortisol in healthy men for utilization in the diagnosis of late-onset hypogonadism in males. Endocr. J. 2009;56:1083–93.
[26] Kutsukake N, Ikeda K, Honma S, Teramoto M, Mori Y, Hayasaka I, et al. Validation of salivary cortisol and testosterone assays in chimpanzees by liquid chromatography–tandem mass spectrometry. Am. J. Primatol. 2009;71:696–706.
[27] Nakamura Y, Rege J, Satoh F, Morimoto R, Kennedy MR, Ahlem CN, et al. Liquid chromatography–tandem mass spectrometry analysis of human adrenal vein corticosteroids before and after adrenocorticotropic hormone stimulation. Clin. Endocrinol. (Oxf.) 2012;76:778–84.
[28] Snoj T, Dolenc J, Kobal S. Sex steroid levels in urine of cattle of different ages: evaluation of abuse control procedures. Food Addit. Contam. Part A Chem. Anal. Control Expo Risk Assess 2014;31:614–20.
[29] Ke Y, Bertin J, Gonthier R, Simard JN, Labrie F. A sensitive, simple and robust LC–MS/MS method for the simultaneous quantification of seven androgen- and estrogen-related steroids in postmenopausal serum. J. Steroid Biochem. Mol. Biol. 2014;144:523–34.
[30] Uzunov DP, Cooper TB, Costa E, Guidotti A. Fluoxetine-elicited changes in brain neurosteroid content measured by negative ion mass fragmentography. Proc. Natl. Acad. Sci. U.S.A. 1996;93:12599–604.
[31] Kim SB, Hill M, Kwak YT, Hampl R, Jo DH, Morfin R. Neurosteroids: cerebrospinal fluid levels for Alzheimer's disease and vascular dementia diagnostics. J. Clin. Endocrinol. Metab. 2003;88:5199–206.
[32] Rasmusson AM, Pinna G, Paliwal P, Weisman D, Gottschalk C, Charney D, et al. Decreased cerebrospinal fluid allopregnanolone levels in women with posttraumatic stress disorder. Biol. Psychiatry 2006;60:704–13.
[33] Starka L, Hill M, Kancheva R, Novak Z, Chrastina J, Pohanka M, et al. 7-Hydroxylated derivatives of dehydroepiandrosterone in the human ventricular cerebrospinal fluid. Neuro Endocrinol. Lett. 2009;30:368–72.
[34] Hill M, Parizek A, Cibula D, Kancheva R, Jirasek JE, Jirkovska M, et al. Steroid metabolome in fetal and maternal body fluids in human late pregnancy. J. Steroid Biochem. Mol. Biol. 2010;122:114–32.
[35] Rosner W, Hankinson SE, Sluss PM, Vesper HW, Wierman ME. Challenges to the measurement of estradiol: an endocrine society position statement. J. Clin. Endocrinol. Metab. 2013;98:1376–87.
[36] Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H. Position statement: utility, limitations, and pitfalls in measuring testosterone: an endocrine society position statement. J. Clin. Endocrinol. Metab. 2007;92:405–13.
[37] Ohlsson C, Nilsson ME, Tivesten A, Ryberg H, Mellstrom D, Karlsson MK, et al. Comparisons of immunoassay and mass spectrometry measurements of serum estradiol levels and their influence on clinical association studies in men. J. Clin. Endocrinol. Metab. 2013;98:E1097–102.
[38] Labrie F, Bélanger A, Bélanger P, Bérubé R, Martel C, Cusan L, et al. Metabolism of DHEA in postmenopausal women following percutaneous administration. J. Steroid Biochem. Mol. Biol. 2007;103:178–88.
[39] Ahboucha S, Talani G, Fanutza T, Sanna E, Biggio G, Gamrani H, et al. Reduced brain levels of DHEAS in hepatic coma patients: significance for increased GABAergic tone in hepatic encephalopathy. Neurochem. Int. 2012;61:48–53.
[40] Caruso D, Scurati S, Maschi O, De Angelis L, Roglio I, Giatti S, et al. Evaluation of neuroactive steroid levels by liquid chromatography–tandem mass spectrometry in central and peripheral nervous system: effect of diabetes. Neurochem. Int. 2008;52:560–8.
[41] Rustichelli C, Pinetti D, Lucchi C, Ravazzini F, Puia G. Simultaneous determination of pregnenolone sulphate, dehydroepiandrosterone and allopregnanolone in rat brain areas by liquid chromatography–electrospray tandem mass spectrometry. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 2013;930:62–9.
[42] Higashi T, Nishio T, Yokoi H, Ninomiya Y, Shimada K. Studies on neurosteroids XXI: an improved liquid chromatography–tandem mass spectrometric method for determination of 5alpha-androstane-3alpha,17beta-diol in rat brains. Anal. Sci. 2007;23:1015–9.
[43] Kenealy BP, Kapoor A, Guerriero KA, Keen KL, Garcia JP, Kurian JR, et al. Neuroestradiol in the hypothalamus contributes to the regulation of gonadotropin releasing hormone release. J. Neurosci. 2013;33:19051–9.
[44] Caruso D, Pesaresi M, Abbiati F, Calabrese D, Giatti S, Garcia-Segura LM, et al. Comparison of plasma and cerebrospinal fluid levels of neuroactive steroids with their brain, spinal cord and peripheral nerve levels in male and female rats. Psychoneuroendocrinology 2013;38:2278–90.
[45] Draisci R, Palleschi L, Ferretti E, Lucentini L, Cammarata P. Quantitation of anabolic hormones and their metabolites in bovine serum and urine by liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 2000;870: 511–22.
[46] Wang C, Catlin DH, Demers LM, Starcevic B, Swerdloff RS. Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography–tandem mass spectrometry. J. Clin. Endocrinol. Metab. 2004;89:534–43.
[47] Soldin SJ, Soldin OP. Steroid hormone analysis by tandem mass spectrometry. Clin. Chem. 2009;55:1061–6.
[48] Kushnir MM, Rockwood AL, Bergquist J. Liquid chromatography–tandem mass spectrometry applications in endocrinology. Mass Spectrom. Rev. 2010;29:480–502.
[49] Bélanger B, Bélanger A, Labrie F, Dupont A, Cusan L, Monfette G. Comparison of residual C-19 steroids in plasma and prostatic tissue of human, rat and guinea pig after castration: unique importance of extratesticular androgens in men. J. Steroid Biochem. 1989;32:695–8.
[50] Guidance for Industry. Bioanalytical Method Validation – Revision 1. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). Division of Drug Information, WO51, Room 2201, Center for Drug Evaluation and Research, Food and Drug Administration. <http://wwwfdagov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/defaulthtm>. 2013.
[51] Zhang F, Bartels MJ, Geter DR, Carr MS, McClymount LE, Marino TA, et al. Simultaneous quantitation of testosterone, estradiol, ethinyl estradiol, and 11-ketotestosterone in fathead minnow fish plasma by liquid chromatography/positive atmospheric pressure photoionization tandem mass spectrometry. Rapid Commun. Mass Spectrom. 2009;23:3637–46.
[52] Szarka S, Nguyen V, Prokai L, Prokai-Tatrai K. Separation of dansylated 17beta-estradiol, 17alpha-estradiol, and estrone on a single HPLC column for simultaneous quantitation by LC-MS/MS. Anal. Bioanal. Chem. 2013;405:3399–406.
[53] Arai S, Miyashiro Y, Shibata Y, Kashiwagi B, Tomaru Y, Kobayashi M, et al. New quantification method for estradiol in the prostatic tissues of benign prostatic hyperplasia using liquid chromatography–tandem mass spectrometry. Steroids 2010;75:13–9.
[54] Inoue T, Miki Y, Abe K, Hatori M, Hosaka M, Kariya Y, et al. Sex steroid synthesis in human skin in situ: the roles of aromatase and steroidogenic acute regulatory protein in the homeostasis of human skin. Mol. Cell. Endocrinol. 2012;362:19–28.
[55] Sasaki Y, Miki Y, Hirakawa H, Onodera Y, Takagi K, Akahira J, et al. Immunolocalization of estrogen-producing and metabolizing enzymes in benign breast disease: comparison with normal breast and breast carcinoma. Cancer Sci. 2010;101:2286–92.
[56] Maeda N, Tanaka E, Suzuki T, Okumura K, Nomura S, Miyasho T, et al. Accurate determination of tissue steroid hormones, precursors and conjugates in adult male rat. J. Biochem. 2013;153:63–71.
[57] McNamara KM, Harwood DT, Simanainen U, Walters KA, Jimenez M, Handelsman DJ. Measurement of sex steroids in murine blood and reproductive tissues by liquid chromatography–tandem mass spectrometry. J. Steroid Biochem. Mol. Biol. 2010;121:611–8.
[58] Methlie P, Hustad S, Kellman R, Almas B, Erichsen M, Husebye E, et al. Multisteroid LC–MS/MS assay for glucocorticoids and androgens and its application in Addisons's disease. Endocr. Connect. 2013;2:125–36.
[59] Santa T. Derivatization in liquid chromatography for mass spectrometric detection. Drug Discov. Ther. 2013;7:9–17.
[60] Higashi T, Nishio T, Hayashi N, Shimada K. Alternative procedure for charged derivatization to enhance detection responses of steroids in electrospray ionization-MS. Chem. Pharm. Bull. (Tokyo) 2007;55:662–5.
[61] Kushnir MM, Rockwood AL, Bergquist J, Varshavsky M, Roberts WL, Yue B, et al. High-sensitivity tandem mass spectrometry assay for serum estrone and estradiol. Am. J. Clin. Pathol. 2008;129:530–9.
[62] Taylor PJ. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray-tandem mass spectrometry. Clin. Biochem. 2005;38:328–34.
[63] Bertin J, Ouellet J, Dury AY, Pelletier G, Labrie F. Expression of the estrogen receptors and steroidogenic enzymes involved in estradiol formation in the monkey vagina. Am. J. Obstet. Gynecol. 2014;211:e1–9.
[64] Bertin J, Dury AY, Ouellet J, Pelletier G, Labrie F. Localization of the androgen-synthesizing enzymes, androgen receptor and sex steroids in the vagina: possible implications for the treatment of postmenopausal sexual dysfunction. J. Sex. Med. 2014;11:1949–61.
[65] Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiol. Rev. 2001;81:741–66.
[66] Bartzokis G. Age-related myelin breakdown: a developmental model of cognitive decline and Alzheimer's disease. Neurobiol. Aging 2004;25:5–18. Author reply 49–62.