J. Biochem. 2013;153(1):63–71 doi:10.1093/jb/mvs121

JB THE JOURNAL OF BIOCHEMISTRY

Accurate determination of tissue steroid hormones, precursors and conjugates in adult male rat

Received August 6, 2012; accepted September 16, 2012; published online October 10, 2012

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The actual levels of steroid hormones in organs are vital for endocrine, reproductive and neuronal health and disorders. We developed an accurate method to determine the levels of steroid hormones and steroid conjugates in various organs by an efficient preparation using a solid-phase-extraction cartridge. Each steroid was identified by the precursor ion spectra using liquid chromatography–electrospray ionization time-of-flight mass spectrometry, and the respective steroids were quantitatively analysed in the selected reaction monitoring mode by liquid chromatograph-mass spectrometry/mass spectrometry (LC-MS/MS). The data showed that significant levels of testosterone, corticosterone and precursors of both hormones were detected in all organs except liver. The glucuronide conjugates of steroid hormones and the precursors were detected in all organs except liver, but sulfate conjugates of these steroids were observed only in the target organs of the hormones and kidney. Interestingly, these steroids and the conjugates were not observed in the liver except pregnenolone. In conclusion, an accurate determination of tissue steroids was developed using LC-MS analysis. Biosynthesis of steroid hormones from the precursors was estimated even in the target organs, and the delivery of these steroid conjugates was also suggested *via* the circulation without any significant hepatic participation.

Keywords: accurate determination/conjugation/LC-MS analysis/metabolism/tissue steroids.

Abbreviations: LC-MS, liquid chromatograph-mass spectrometry; SRM, selected reaction monitoring.

Steroid hormones are synthesized from cholesterol, and many are of great clinical importance (*1*). The measurement of steroid profiles indicates an important physiological state in endocrine systems, and it has been newly applied in the diagnoses and treatments of congenital adrenal hyperplasia, adrenal insufficiency, chronic pelvic pain and prostatitis, oncology (breast cancer) and athletic competition. Accurate assay methods for the determination of tissue steroid levels are required for the clinical evaluation of a number of common endocrine disorders. Radioimmunoassays (RIAs) are commonly used for the determination of the actual levels of testosterone (TS) (*2*). As RIA methods have the inherent limitations in specificity, it is difficult to determine accurately the low levels of TS present in females and children (*3*, *4*). Gas chromatography-mass spectrometry is used for the determination of low levels of natural steroids with excellent specificity and sensitivity (*5*). However, this method usually requires extra steps for sample preparation and cleanup and is often encountered with the problems in the thermal stability of the steroid derivatives. Recently, several liquid chromatograph-mass spectrometry (LC-MS) methods have been developed for the simultaneous determination of low levels of steroids. The chemical derivatization using a number of reagents, such as dansyl chloride and 2-hydrazino-4-(trifluoromethyl)-pyrimidine enhance the sensitivity of the LC-MS determination of steroids in various biological samples (*6*, *7*). However, for accurate determination of both steroid hormones and glucuronide and sulfate conjugates simultaneously in organs containing various matrixes, an analysis should be conducted without the removal of the matrix, which reduces the ionization efficiency of electrospray ionization (ESI) in LC-MS/MS analysis. A simultaneous analysis was performed on steroid hormones and the conjugate forms in urine and water containing trace amounts of matrix (*8*–*11*). It has generally been proposed that the presence of interference peaks co-existing in biological matrixes and co-eluting with the analyte of interest caused ionization suppression, reducing the detection sensitivity (*12*, *13*). For the measurement of steroids in organs, LC-MS analysis has several advantages over immunoassays, including a better specificity and the ability to quantify numerous steroids in a single run (*14*, *15*). Especially, liquid chromatography–electrospray ionization time-of-flight mass spectrometry (LC-TOFMS) made possible to perform the accurate mass determination of components containing matrixes. This method could be applied to drug screening using ESI to produce pseudomolecular ions, measuring their mass and comparing the results with a database containing the exact monoisotopic masses of the target analysis (*16*, *17*). The difference between the theoretical and measured isotopic patterns provides the evaluation and

numerical expression using the SigmaFit algorithm for exact identification. For example, it has been demonstrated to aid in the identification and to reduce the incidence of false-positive findings in human urine drug screening when combined with mass accuracies of <10 ppm using this method (*18*).

In this study, we developed accurate and highly sensitive assay methods for various steroids and their glucuronide and sulphate conjugates using LC-TOF MS and LC-MS/MS, and obtained the actual levels of steroid hormones and precursors in various organs. Based on our results, we discussed about metabolism and the possibility of steroid biosynthesis and conjugate reactions in each organ.

Materials and Methods

Chemicals and reagents

The following were purchased from Sigma-Aldrich (St. Louis, MO, USA): steroid hormones—TS, 17α and 17β-estradiol (17α, 17β-E2); precursors—pregnenolone (PGN), progesterone (PGT), 17-hydroxyprogesterone (HPGT), androstenedione (ADS), deoxycorticosterone (DCC) and corticosterone (CCS); conjugated metabolites—TS-17(β-D-glucuronide) (TS-17G), 17β-estradiol-3(β-D-glucuronide) (E2-3G) and 17β-estradiol-3-sulphate(E2-3S); and stable isotopes—TS-d_3 and 17β-estradiol-d_2 (E2-d_2). Triethylamine was purchased from Wako Pure Chemical Industries (Osaka, Japan). Methanol, acetonitrile and hexane for pesticide residue analysis and citric acid were purchased from Kanto Chemical (Tokyo, Japan). LC-MS grade acetonitrile, formic acid and ammonium hydroxide solution were purchased from Supelco (Bellefonte, PA, USA). β-Glucuronidase (Type B-1) and sulphatase (Type H-1) were purchased from Sigma-Aldrich.

Preparation of rat organs

Male Sprague–Dawley rats (weight: 280 ± 20 g; age: 8–10 weeks) were fed, housed and allowed to adapt to their environments for 1 week before the experiments. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The blood and organs were prepared from the animals by exsanguination under isoflurane anaesthesia. After dissection, the organ samples excised post-mortem were weighed, immediately frozen and stored at −25°C until use.

Preparation of samples for MS analysis

The samples were weighed in 50 ml centrifuge tubes. Then, 5.0 ng/ml TS and E2 of the internal standard were added to the tube. A 15-ml aliquot of acetonitrile was added, and the samples were homogenized for 1 min using a biotechnology mixer. The mixture was centrifuged at 10,160 × g for 10 min at 4°C, then 30 ml of hexane was added to the supernatant and the tube was shaken for 5 min at high speed. The acetonitrile layers were collected, and 0.3 ml formic acid was added. An Oasis Weak Anion Exchange (WAX) 150 mg solid-phase-extraction (SPE) cartridge (Waters Midford, MA, USA) was conditioned using 5 ml methanol, followed by acetonitrile containing 2% formic acid. The sample was loaded onto the WAX SPE cartridge at ~3–5 ml/min (Sample 1). After loading, the waste sample collected *via* the Oasis WAX SPE cartridge was linked in-series to a Hybrid SPE Phospholipid cartridge (Supelco) (Sample 2). Sample 1 was collected in another bottle *via* the Oasis WAX SPE cartridge, which was loaded with 20 ml methanol, washed and then immediately loaded with 10 ml 10 mM triethylamine. The liquid was evaporated and solubilized with 10 ml 40 mM sodium acetate buffer (pH adjusted to 4.5 after the solubilization); 0.1 ml 2.5 mg/ml β-glucuronidase or sulphatase was added (incubation for 30 min at 37°C), and 10 ml acetonitrile was added, evaporated and solubilized with 25 ml of dichloromethane and 50 ml 5% sodium chloride. The dichloromethane layer was evaporated and solubilized with 0.2 ml methanol, and the steroids and conjugates were then assayed by LC-TOF MS and LC-MS/MS analyses. Sample 2 was collected for loading onto the Hybrid SPE Phospholipid cartridge, with 5 ml methanol and 5 ml acetonitrile with 2% citric acid then added. The collected extract was subsequently evaporated, and the leftover residue was dissolved in 1 ml methanol and then centrifuged at 17,390 × g for 5 min using PVDF 0.22 μm Ultrafree-MC (Millipore, Billerica, MA, USA). The supernatant was collected for the LC-TOF MS and LC-MS/MS analysis as shown in Fig. 1.

Determination of steroids expressed in the tissues

The HPLC system was a UFLC Nexera (Shimadzu, Japan) instrument, comprising a vacuum degasser, autosampler, binary pump and column oven. The separation was achieved using an L-column 2 [C_{18} 2.1 × 150 mm 2-μm particle size, Chemicals Evaluation and Research Institute (CERI), Japan] at a 200 μl/min flow rate at 40°C. A guard column with the pre-column filter (L-column pre-column filter 0.5 μm, CERI, Japan) was used for the analysis. A 10-μl aliquot was used for the autosampler injection. The positive ion mode scanning of a gradient mobile phase consisting of (A) 0.1% formic acid solution and (B) acetonitrile with 0.1% formic acid solution was used. For the gradient elution, (A)/(B) ratios were used from 95/5 to 40/60 and 40/60 to 5/95 for between 0 and 3 min and between 3 and 9 min, respectively, followed by a 2 min hold at 95% (B) and a final return to 95% (A) within 4 min. The negative ion mode scanning of a gradient mobile phase consisting of (A) 0.03% ammonium hydroxide solution and (B) acetonitrile with 0.03% ammonium hydroxide solution was also used. The same positive ion mode was used for the gradient pattern. The mass analyser was a microTOF-QII time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with an orthogonal ESI source and a 6-port diverter valve. The instrument was operated in positive ion or negative ion mode using a range of 50–1,000 m/z. The capillary voltage of the ion source was set to 4,500 V, then the nebulizer gas flow was 1.6 bar and the dry gas flow was 8 l/min. The dry temperature was set to 180°C. Instrument calibration was performed prior to each sequence using 10 mM sodium formate/2-propanol (1:1, v/v). The post-run internal mass scale calibration for the individual samples was performed using data acquired during a calibration injection at the beginning of the run *via* a 6-port diverter valve equipped with a 20-μl loop. The calibrant was also injected at the end of each run for the verification of the calibration stability. The instrument calibration and post-run internal mass scale calibration were performed using sodium formate ions Na (NaCOOH) 1–14 ranging from 90.9766 to 974.8132 m/z with an accuracy of 5 ppm. The data processing was performed using Data Analysis software (version 4.0, Bruker Daltonics). The base mass peak (after background subtraction) was measured after the proton subtraction in the compounds (10 ppm tolerance). For each retrieved chemical formula, the mass error (difference between the measured and theoretical masses) and SigmaFit [a parameter, calculated by the Bruker software, accounting for the difference between the theoretical and measured isotonic pattern; the lower is the sigma value (usually <0.05), the better the matching is] were calculated.

Quantification of steroids expressed in tissues

For the quantification of the steroid hormones and conjugated metabolites, a TSQ Quantum Ultra triple-stage quadrupole mass spectrometer connected to an Ultimate 3,000 (Thermo Fisher Scientific, San Jose, CA, USA) and an ESI ion source device was constructed (LC-MS/MS). Separation was achieved using an L-column 2 [C_{18} 2.1 × 150 mm 3-μm particle size (CERI)] at a 200-μl/min flow rate at 40°C. A guard column with packing material was used as the analytical column. A 10-μl aliquot was used for the autosampler injection. A gradient mobile phase consisting of (A) 0.03% ammonium hydroxide solution and (B) acetonitrile with 0.03% ammonium hydroxide solution was used. In particular, the positive ion microanalysis of original TS and CCS conjugate were used with a mobile phase consisting of (A) 0.1% acetic acid and (B) acetonitrile with 0.1% acetic acid solution. The gradient pattern was the same as for the LC-TOF MS described earlier. The total run time for each sample analysis was 15 min, and the data were collected between 3 and 13 min; the column effluents before 3 min and after 13 min were diverted as waste. The MS system and data were operated and analysed using Xcalliber and LCquan 2.6 software, respectively. The mass spectrometer was operated with positive and negative ionization mode switching. The instrumental parameters were optimized during the direct infusion of the compounds with solvent consisting of 50% (0.1% acetic acid in water/acetonitrile at 1:1 [v/v]) at a flow

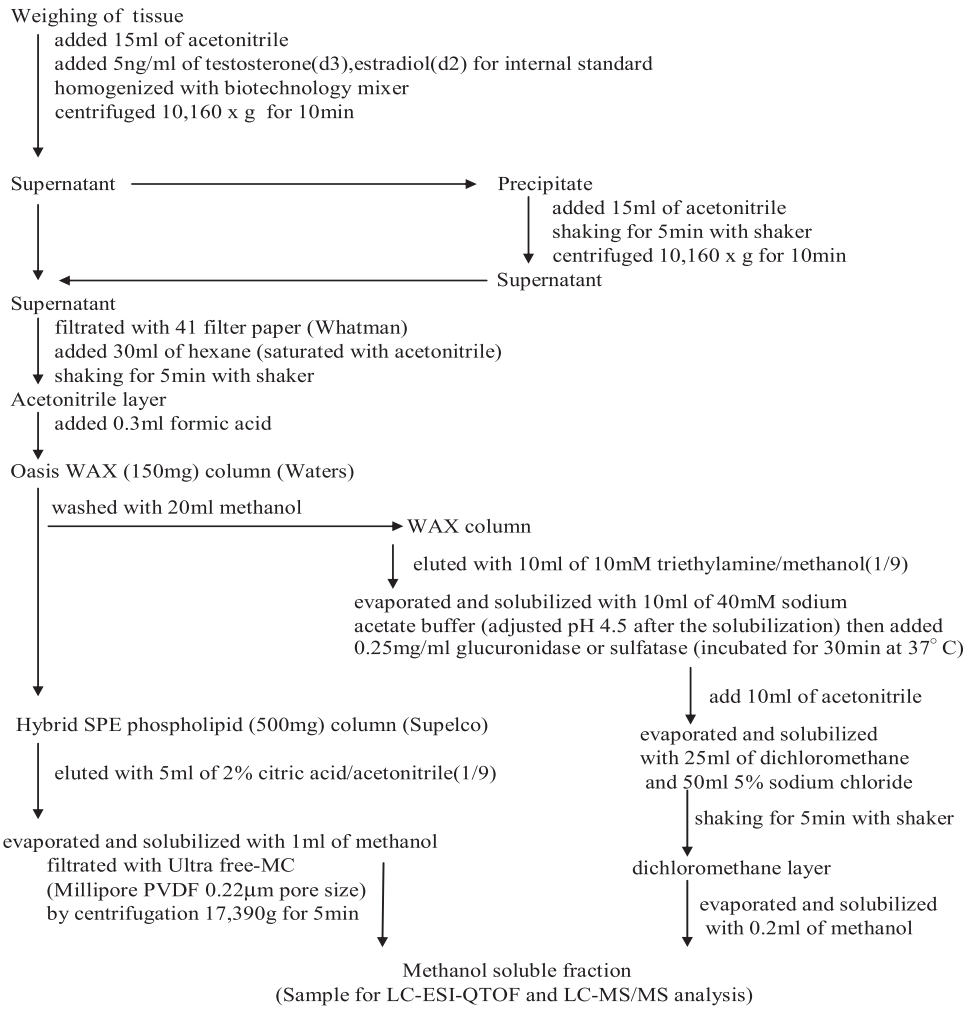

Fig. 1 Preparation of lipophilic steroids and hydrophilic conjugate forms of the steroids from various organs for MS analysis.

rate of 5 μl/min. The $[M+H]^+$, $[M\text{-}H_2O]^+$ and $[M-H]^-$ ions of the compounds were identified by LC-MS, with Q1 operated in the full scanning mode in the range of 50–800 m/z. A product ion spectrum was obtained for each compound. Selected reaction monitoring (SRM) was used for the quantitative analysis (Fig. 3C and D). Nitrogen was used for the sheath gas pressure (setting 50), ion sweep gas pressure (setting 10) and auxiliary gas pressure (setting 15), whereas argon was used as the collision gas for the collision-induced dissociation conditions (setting 1.5 mTorr). The ion spray voltage was set to a positive mode at 3,500 V or a negative mode at 2,500 V. The vaporizer gas temperature was set at 450°C, and the ion transfer capillary temperature was set at 270°C. The tube lens and collision energy for each SRM transition were set.

Calibration curves

Stock solutions of the steroid hormones and conjugated metabolites were used to prepare working standards of the steroid hormones and conjugated metabolites (at concentrations of 0.1, 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 ng/ml) by serial dilution in methanol; the isotopes of the internal standards TS-d_3, E2-d_2 were prepared at a concentration of 5.0 ng/ml. The calibration curves were plotted using the concentration ratios of the analytes to the internal standards (PGN, PGT, HPGT, ADS, TS, DCC, CCS/TS-d_3, 17α and 17 β-E2/E2-d_2) as the x axes and the peak area ratios of the analytes to the internal standards as the y axes. The calibration curves for the steroid hormones and conjugated metabolites showed excellent linearity over the concentration range used ($R^2 > 0.998$), and the detection limits of these were shown in Table I (*19*). The steroid hormones and their conjugated metabolites were used as the standard in the addition method (*20*).

Overall method recovery

Recovery tests were performed to search for the recovery rates and assess the accuracy of the method. Several concentration mixtures of the standards and internal TS-d_3 and E2-d_2 standards were added to 5.0 ng/ml. The samples were measured and evaluated for the recovery rate and relative standard deviation (RSD%) using LC-MS/MS. The accuracy and precision of the entire analytical procedure were evaluated by spiking the whole-organ samples ($n = 3$–5) with 0.1, 0.5, 1.0, 5.0 and 10.0 ng/ml or g at each concentration before using SPE with the working solution. The amount of endogenous steroid hormones and conjugated metabolites were subtracted from the spiked amounts of the analytes in the blood, testis and liver.

Results

Determination of steroids in organs

A simple method for preparing samples for LC-MS analysis with a high sensitivity and accuracy was improved to determine lipophilic compounds, such as TS in the blood and various tissues of rats. The total preparation scheme is shown in Fig. 1. The water-soluble compounds, such as the glucuronide and sulphate conjugates of the steroid hormones, were adsorbed onto an Oasis WAX column, and these compounds were eluted with 10 mM triethylamine methanol (1:9) solution. Because the androgens and estrogens passed through the HybridSPE Phospholipid column, only

Table I. Recovery tests of additive steroids and glucuronides into the blood and the testis in the determination method developed in this study.

		Blood (0.50 ml)[a]		Testis (1.00 g)[a]		Liver (1.00 g)[a]		
Steroids	**Added (ng/g)**	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	**LOD (fmol/g)**
PGN	10.0	93.3	18.5	98.3	7.9	86.7	3.5	
	5.0	91.0	18.4	104.3	14.8	85.0	15.3	14.2
	1.0	85.9	15.7	91.6	19.4	99.3	9.8	
PGT	10.0					90.7	10.9	
	5.0	94.6	8.2	92.0	10.5	86.6	16.0	
	1.0	90.1	6.4	97.7	16.5	107.1	15.3	3.2
	0.1	82.8	4.5	101.6	9.3			
HPGT	10.0					72.4	4.3	
	5.0	110.3	2.3	105.2	7.0	75.8	19.8	
	1.0	86.0	9.4	89.4	10.4	89.7	6.7	2.8
	0.1	97.2	13.6	92.6	16.4			
ADS	10					105.2	7.0	
	5.0	113.6	6.4	86.0	4.5	89.4	10.4	
	1.0	115.4	2.5	86.2	5.6	92.6	16.4	2.6
	0.1	100.7	7.6	102.0	5.3			
TS	10.0					98.6	4.5	
	5.0	106.8	5.2	80.5	4.7	88.0	9.1	
	1.0	104.8	5.7	77.6	5.7	74.3	4.4	0.6
	0.1	110.6	7.8	96.4	15.1			
17α-Estradiol	10.0					108.7	6.0	
	5.0	91.9	14.1	108.0	1.0	89.7	20.5	
	1.0	73.3	2.1	109.5	4.0	89.0	7.9	25.3
	0.5	89.4	15.6	107.0	6.1			
17β-Estradiol	10.0					99.3	3.1	
	5.0	99.3	3.1	105.7	0.7	73.3	2.1	
	1.0	73.3	2.1	81.9	1.5	112.3	6.7	24.2
	0.5	112.3	6.7	96.9	18.4			
CCS	10.0					101.3	4.0	
	5.0	82.7	9.4	84.8	16.4	88.9	9.6	
	1.0	80.7	4.3	100.6	13.9	104.3	2.9	0.4
	0.1	107.8	5.1	85.7	9.1			
11-DCC	10.0					95.9	5.2	
	5.0	78.6	7.4	116.9	1.7	97.6	2.0	
	1.0	90.0	11.3	78.1	4.3	100.7	21.5	9.2
	0.1	81.5	8.8	75.3	1.2			
TS-17-glucuronide	10.0					85.0	8.6	
	5.0	91.8	18.8	85.0	2.6	95.4	16.4	34.6
	1.0	98.5	13.8	95.4	6.4	110.0	10.1	
	0.5	71.3	14.9	117.0	1.1			
17β-Estradiol-3-glucuronide	10.0					103.5	4.3	
	5.0	90.7	10.9	102.7	2.3	97.8	4.0	73.3
	1.0	86.6	16.0	109.2	7.7	119.1	0.5	
	0.5	107.1	15.3	79.1	12.3			
17β-Estradiol-3-sulfate	10.0	90.3	17.1	106.4	1.4	70.2	6.5	
	5.0	101.3	4.6	101.5	13.5	72.3	12.3	146.6
	1.0	82.3	13.2	91.3	12.5	71.2	15.3	

Standard substrates ~10-fold amount of the assay limits were added into the blood, liver and the testis. Standards substrates were extracted and determined by the assay method developed in this study. Recovery data obtained were shown with RSD. The accurate data having 70–120% recovery and RSD under 25% and LOD (signal/noise $[S/N] > 3$) were obtained, respectively, as previously indicated by FDA as a guideline (FDA 2001).

[a]Data presented mean values of $n = 3$–5.

the corticoids and phosphatidylcholine, which inhibits the ESI in LC-MS analysis, were adsorbed to the column. Only the corticoids were eluted from the column with a 2% citrate solution as shown in Fig. 2. Using both columns allowed for the simultaneous analysis of the lipophilic steroid hormones and hydrophilic glucuronide and sulphate conjugates with a high degree of sensitivity and recovery as shown in Table I. The recovery tests for TS and the glucuronides were performed as described in the Materials and Methods section. High-accuracy results were obtained and showed significant trueness (70–120% recovery) with a high precision. All the substrate peaks in the LC-MS analysis indicated significant S/N values of <10. The quantitation limits of these substrates were determined and are shown in Table I (e.g. 1.0 ng of TS/g of liver and 0.1 ng of TS/ml of blood). This removal of phosphatidylcholine is a critical step for obtaining a highly sensitive and accurate LC-TOF MS analysis. Each steroid formula in the organs was detected using SigmaFit (Fig. 3). For example, the measured isotopic pattern of the positive ion of TS $[C_{19}H_{28}O_2]^+$ found in rat testis was identified using the developed method (Fig. 3A) and the theoretical isotopic abundance (Fig. 3B). The SigmaFit algorithm compares both the mass distances between the ions

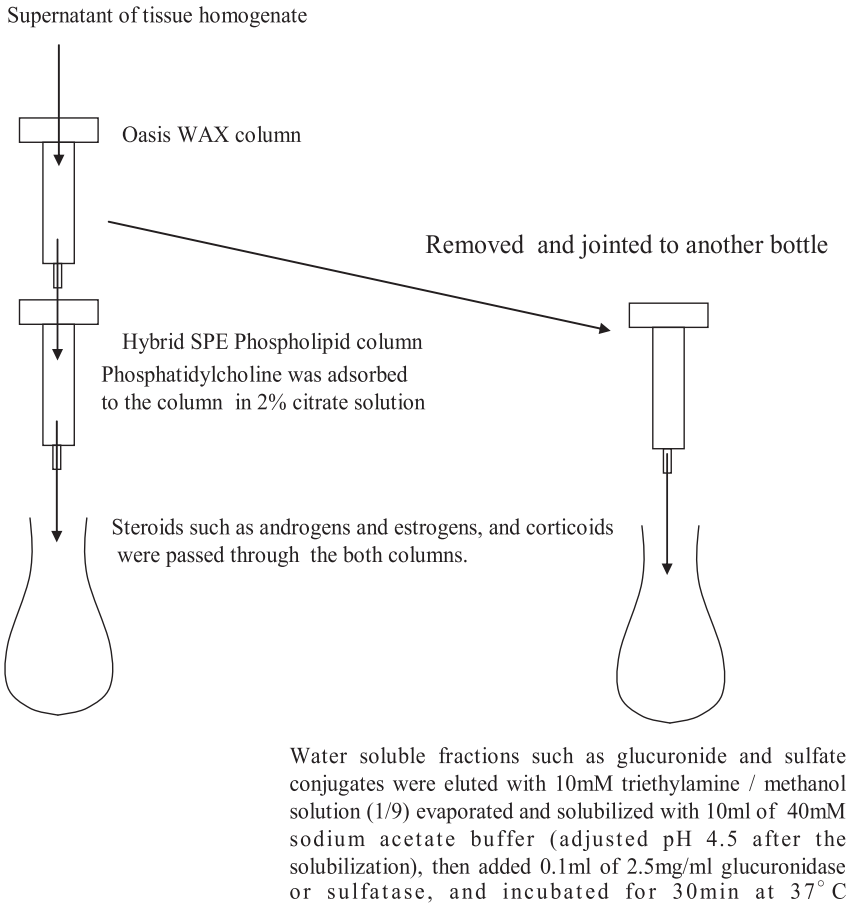

Fig. 2 Critical steps for separation of lipophilic steroids and hydrophilic conjugate forms of the steroids and removing various contaminants from the extracted preparations of organs.

and their relative peak intensities with those predicted and expresses the degree of fit as a δ value between 0 and 1; a low δ value indicates a closer match (*18*, *21*). In this case, the delta value was 0.0118, and the mass error was 2 mDa. Similarly, the identification of the steroid hormones was performed using the SigmaFit algorithm, and the identified steroids were then quantified by LC-MS/MS analysis (*22*). Other steroids were identified by same procedure as shown in Supplementary Fig. S3.

Tissue distribution of steroids

The steroid concentrations in various organs were assayed using the MS procedure developed in this study, and the results are shown in Table II. The data showed that TS and CCS were distributed in all of the organs tested, and these two steroid hormones are known to play multiple roles in the respective organs and regulate the endocrine system in the entire body. Significant levels of two of the precursors for steroid hormone biosynthesis, PGT and 11-DCC, were also observed in the blood and all of the organs (Table II). This result suggests that the endo products TS and CCS and also the precursors were transported to the various target organs *via* the circulation from the endocrine organs. Interestingly, only a trace amount of CCS and PGN were detected in the liver before the removal of blood by perfusion with PBS (Supplementary Figs S1 and S2), but only PGN and no other steroids were detected in the liver after the perfusion (Table II). The levels of the two conjugate forms of various steroids in the organs are shown in Table III. The glucuronide conjugates of endo products, TS and CCS and all of the precursors, except PGN, were observed in the testis and adrenal gland. Higher levels of CCS and its precursors were observed in the adrenal gland (Table II), and the glucuronide forms were also detected at higher levels in the same organs (Table III). In contrast, the sulphate conjugates of the endo products and a portion of the precursors were observed only in the blood and hormone target organs, such as the brain, muscle and kidney (Table III). Both of these conjugation reactions may be performed in the respective organs, and/or the conjugates were transported *via* blood circulation from the endocrine organs. It is very interesting that steroid hormones and the glucuronide and sulphate conjugate forms were not detected in the liver (Table III).

Discussion

Steroid assays play an important role in the clinical evaluation of a number of common endocrine disorders, and LC-MS analysis has several advantages

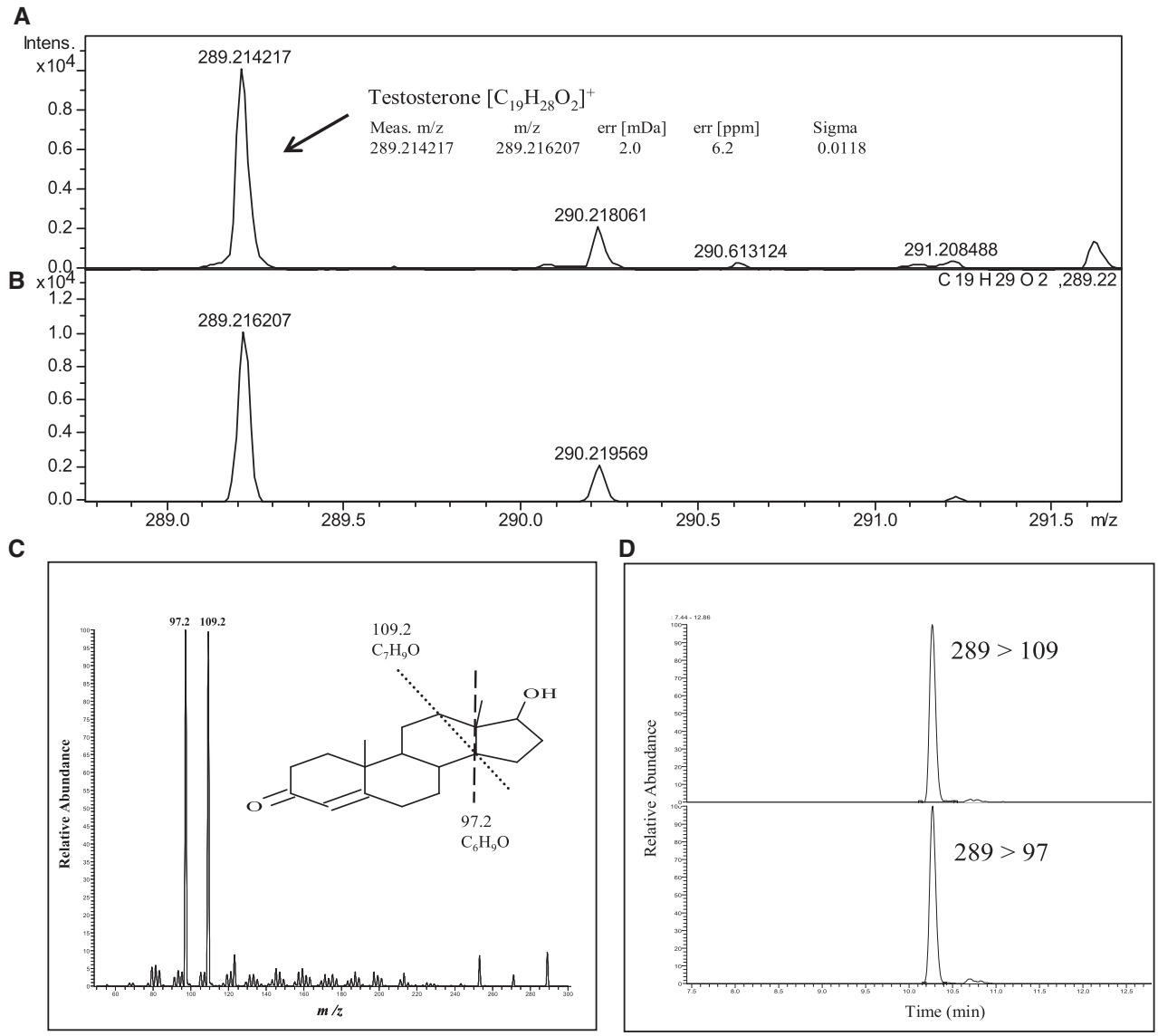

Fig. 3 Identification and determination of steroids by the SigmaFit algorithm and LC-TOF MS analysis. The chromatograms of the preparations from the testis by TOF MS analysis developed in this study (**A**) and theoretical abundances of testosterone containing an isotope (**B**). A δ-value was calculated by the program 'sigmaFit algorithm' after comparing the two chromatograms A and B. The δ-value between 0 and 1.0 shows higher identification. In this case, small δ-value was observed at 0.0118 and the mass error was only 2.0 mDa showing that TS in the sample was identified. The identification of other steroids was performed by the same procedure using sigmaFit algorithm. Identically, TS precursors were identified from testis. Product-ion spectrums were obtained for TS by MS/MS analysis (**C**). SRM of TS was used for quantitative analysis as described in Materials and Methods section (**D**).

over immunoassays for the measurement of steroids, such as an improved specificity and ability to quantify numerous steroids. To analyse both steroid hormones and water-soluble glucuronide and sulphate conjugates simultaneously in organs containing various matrices, an analysis should be conducted without the derivatization of the specimen (*23*) and with the removal of the matrix, which reduces the ionization efficiency of the ESI in LC-MS/MS analysis. An simultaneous LC-MS analysis was performed on the steroid hormones, glucuronide and sulphate conjugates contained in urine and water, with only trace amounts of matrix (*6–9*). We developed an accurate method to determine various steroids and also their conjugate forms in tissues using two different columns. The limitation of detection (LOD) in case of serum TS were reported previously as 920 fmol/ml by RIA (*3*), 600 fmol/ml by Gas-mass (*5*) and 50 fmol/ml by LC-mass (*3*), and the LOD value of our method developed in this study was 0.6 fmol/ml (Table I), corresponding ~1,500-fold sensitivity of that using RIA. Neurosteroids and their precursors and metabolites in whole brain were identified and determined, however, some precursors and sulfate conjugates could not be detected by the method using Gas-mass analysis (*24*). In non-reproductive organs, such as the brain, the synthesis of steroid hormones has been shown through the detection of cytochrome P450scc and the mRNAs encoding the enzymes that synthesize steroid hormones. Because of the broad specificity of antibodies against small molecules, the accurate determination of steroid levels in organs is difficult using RIA

Table II. Steroid hormones and the precursors in the various organs of adult male rats.

						Brain			
Steroids	**Blood**	**Testis**	**Adrenal glands**	**Liver**	**Kidney**	**Cerebrum**	**Cerebellum**	**Hippocampus**	**Muscle**
TS	5.4 ± 1.3	175.8 ± 41.8	0.105 ± 0.004	–	8.5 ± 1.5	9.1 ± 2.6	9.4 ± 2.5	1.17 ± 0.21	3.14 ± 0.90
ADS	0.57 ± 0.16	45.6 ± 8.8	0.15 ± 0.02	–	6.1 ± 2.7	–	–	0.052 ± 0.015	0.88 ± 0.09
HPGT	–	36.2 ± 9.5	–	–	–	–	–	–	–
PGT	2.21 ± 1.43	13.7 ± 5.3	28.5 ± 2.8 *a	–	10.1 ± 0.4	15.67 ± 5.40	4.4 ± 0.36	0.083 ± 0.043	0.45 ± 0.15
PGN	–	25.4 ± 2.5	4.99 ± 0.86 *a	304.2 ± 55.1	25.3 ± 4.4	–	–	–	216.2 ± 28.0
11-DCC	20.4 ± 3.1	40.2 ± 17.8	17.2 ± 1.5 *a	–	30.4 ± 1.6	11.2 ± 5.9	18.8 ± 1.0	0.368 ± 0.07	14.4 ± 3.4
CCS	140.3 ± 38.1	137.7 ± 24.1	38.2 ± 1.1 *a	–	414.7 ± 127.8	101.0 ± 6.6	92.7 ± 6.5	2.55 ± 0.62	57.0 ± 19.3

Concentration of steroids and the precursors in rat organs were assayed by the method developed in this study as described in 'Materials and Methods' section. Data were shown as the means ± SD for 3–5 animals (nmol/ml or g of tissue weight). '*a' and '–' mean as 'μmol/g' and 'LOD', respectively.

Table III. Glucuronide or sulfate-conjugated steroids in various organs adult male rats.

Glucuronide conjugates	**Blood**	**Testis**	**Adrenal glands**	**Liver**	**Kidney**	**Whole brain**	**Muscle**
TS	0.039 ± 0.009	0.023 ± 0.008	–	–	0.037 ± 0.020	0.024 ± 0.006	0.062 ± 0.005
ADS	–	0.021 ± 0.005	–	–	–	–	–
HPGT	–	0.052 ± 0.004	–	–	–	–	–
PGT	–	0.050 ± 0.012	4.39 ± 2.30	–	–	0.036 ± 0.012	–
PGN	4.39 ± 0.55	2.69 ± 1.42	5.71 ± 2.17	8.18 ± 3.81	9.83 ± 1.00	7.84 ± 0.46	6.99 ± 1.59
11-DCC	–	–	4.55 ± 1.84	–	–	0.060 ± 0.028	–
CCS	0.077 ± 0.007	0.021 ± 0.016	6.07 ± 3.19	–	0.063 ± 0.010	0.070 ± 0.054	0.028 ± 0.015
Sulfate conjugates	**Blood**	**Testis**	**Adrenal glands**	**Liver**	**Kidney**	**Whole brain**	**Muscle**
TS	0.068 ± 0.006	–	–	–	0.041 ± 0.018	0.015 ± 0.011	0.032 ± 0.002
ADS	–	–	–	–	–	–	–
17α-HPGT	–	–	–	–	–	–	–
PGT	–	–	–	–	0.042 ± 0.006	0.013 ± 0.005	–
PGN	6.12 ± 0.42	–	–	17.95 ± 3.50	15.99 ± 5.98	10.91 ± 1.43	8.66 ± 0.73
11-DCC	–	–	–	–	–	0.012 ± 0.004	–
CCS	0.056 ± 0.002	–	–	–	0.067 ± 0.017	0.018 ± 0.008	0.036 ± 0.018

Tissue-conjugated steroids were assayed by the method developed in this study as shown in 'Materials and Methods' section. Data were shown as the means ± SD for 3–5 animals (nmol/ml or g of tissue weight). '–' mean as 'LOD'.

and ELISA (*25*, *26*). We have developed a method for the determination of steroids using an efficient preparation of organs with two columns, with the determination being performed using LC-TOF MS and LC-MS/MS analysis; highly accurate results were obtained (Table II). Serum TS in pregnant and immature rats could be detected by our method developed in this study, and they were very low levels (means ± SD; 0.11 ± 0.06 and 0.18 ± 0.08 nmol/ml, respectively), corresponding ~2–4% of that in adult male rat (Table II). Previously, sex steroids were thought to originate exclusively from the gonads and adrenal glands; however, it is now accepted that local steroid synthesis occurs in a number of tissues, such as adipose tissue, the cardiovascular system and the brain (*27*). In adult male rat neurons, significant localization and protein expression were demonstrated for both cytochromes P45017α and P450 aromatase by means of immunohistochemical staining, western blot analysis and RT-PCR (*28*). The presence of a conversion activity from PGN to estradiol through TS was also observed. Estradiol synthesis has been demonstrated in cultured rat hippocampus slices and dispersed cells (*29*). In this study, we could additively confirm the presence of a biosynthesis system for neurosteroids in rat hippocampus by the new detection of steroid precursors, such as ADS and PGT (Table II). Rat Leydig cells expressing 11β-hydroxylase have been demonstrated using RT-PCR, western blot analysis and immunohistochemistry (*30*), and it was suggested that the enzyme may be involved in the regulation of glucocorticoid metabolism within the testis through the local biosynthesis of endogenous inhibitors, 11β-hydroxylated steroids, of 11β-HSD1 CCS metabolism (*31*, *32*). However, 11β-hydroxylase catalyses the conversion of 11-DCC to CCS, and we found a significant level of 11-DCC in the testis (Table II), indicating the possibility of the biosynthesis of CCS *via* the 11β-hydroxylation of the 11-DCC detected in rat testis. We also observed significant levels of 11-DCC in the brain, suggesting the possibility of CCS biosynthesis in the brain, as previously reported in the hippocampus (*33*). Recently, neurosteroid glucuronides have been found in mouse brain by LC-MS analysis, and the authors showed the formation of those glucuronides *in vitro* using a brain homogenate S-9 fraction (*34*). It is interesting that the adrenal steroids, CCS, 11-hydroxycorticosterone and PGT, were observed at

high concentrations, and the conjugate forms were also found in higher levels in the adrenal gland (Tables II and III), suggesting that each steroid was glucuronidated after the synthesis in the adrenal gland, as demonstrated in the brain (*34*). The glucuronide forms of the adrenal steroids CCS and 11-DCC were detected in the blood and whole organs at high concentrations (Table III), suggesting that these glucuronides were delivered to the target cells that express a glucuronide transporter and that the steroid can perform its respective function after deconjugation. Neurosteroid sulphates have not been previously found in the rodent brain (*35*, *36*), however, sulphate conjugates of PGT, 11-DCC, CCS and TS were detected in the brain at very low levels (10–20 pmol/g) (Table III) in this and earlier studies (*37*, *38*). Because the glucuronides and sulphates of major steroids were detected in the blood and kidney at similar levels, these conjugates were excreted into the urine *via* the kidneys. Our results suggested that the glucuronide and sulphate conjugates are the excreted forms of steroid hormones and also the transported forms, as previously reported with regard to estradiol sulphate circulation (*39*). These steroid hormones, their precursors and conjugate forms were not detected in the liver perfused with PBS except PGN (Tables II, III; Supplementary Data). A further comprehensive computer search of the steroid metabolites of phases I and II in the MS spectra was performed using the software 'metabolitetools' (Version 2.0, Bruker Daltonics Co.), but no corresponding peaks were observed in the liver.

We conclude the following: (i) steroid hormones were delivered from the endocrine organs to the target organs, together with the many precursors, which may then be converted to steroid hormones in each organ; (ii) those steroids were glucuronidated after their biosynthesis in the hormone-producing organs as delivery forms; and (iii) interestingly, these metabolisms were performed without any significant hepatic participation.

Supplementary Data

Supplementary Data are available at *JB* Online.

Acknowledgements

This work was supported by Grant-in-aid and Support Project to Assist Private Universities in Developing Bases for Research from the Ministry of Education, Science, Sports and Culture and Technology in Japan. The authors are grateful to Mr Michio Sasaki of the Association of Meat Science and Technology Institute for his helpful suggestions.

Conflict of Interest
None declared.

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