A mass spectrometric method for sensitive profiling of multi-class steroid hormones

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Abbreviations

11-OH-An	11-β-hydroxyandrosterone
16-keto-E2-d_5	16-keto-17β-estradiol-d_5
16-OH-E1	16-hydroxyestrone
17-OH-P4	17α-hydroxyprogesterone
17-OH-P5	17α-hydroxypregnenolone
3-OH steroids	steroids that contain a 3-hydroxyl group
7-OH-DHEA	7α-hydroxydehydroepiandrosterone
7-OH-P5	7α-hydroxypregnenolone
ACN	acetonitrile
AcOH	acetate acid
AE	androstenedione
al-P5	allopregnenolone
An	androsterone
APD	alphadolone
AT	adrenosterone
CID	collision induced dissociation
COB	corticosterone
COB-d_4	corticosterone-9,11,11,12-d_4
COL	Cortisol
COR	Cortisone
COS	11-deoxycortisol
DHEA	dehydroepiandrosterone
DHEA-d_5	dehydroepiandrosterone-2,2,3,4,4-d_5
DHT	dehydrotestosterone
DOC	11-Deoxycorticosterone
E1	estrone
E2	estradiol
E2-d_5	Estradiol-d5
E3	estriol
ESI	electrospray ionization
EtOAc	ethyl acetate
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LiCl	lithium chloride
LiOAc	lithium acetate

LLE	liquid–liquid extraction
MeOH	methanol
MRM	multiple reaction monitoring
NaCl	sodium chloride
P4	progesterone
P4-$^{13}C_3$	progesterone-2,3,4-$^{13}C_3$
P5	pregnenolone
P5-$^{13}C_2$,d_2	pregnenolone-$^{13}C_2$,d_2
SPE	solid-phase-extraction
TE	testosterone
THB	tetrahydrocorticosterone
TH-COL	tetrahydrocortisol
TH-COR	tetrahydrocortisone
TH-COR-d_6	tetrahydrocortisone-d_6
TH-DOC	3β,5α-tetrahydrodeoxycorticosterone
THS	tetrahydrodeoxycortisol

General Introduction

Steroids influence brain development, behavior, cognition, neuroplasticity and neuroinflammation, and can be synthesized within the brain from a cholesterol backbone (a planar tetracyclic ring) [1-3]. Steroid hormones are enzymatically transformed into different classes of steroids, including estrogens (female reproductive steroids), androgens (male reproductive steroids), progestogens (pregnancy steroids), and corticosteroids (stress steroids) [4].

1. The biological function and syntjetic pathway of steroids

Neurosteroids, synthesized by the brain and nervous system in adrenals, gonads and the placenta, have variety of profound functions in the brain. Steroids play a pivotal role in mediating many brain functions by effecting through the regulation of neurotransmitter receptors or through the change of intracellular signaling cascades [5]. The metabolic pathways of steroids were shown in Fig. 1.

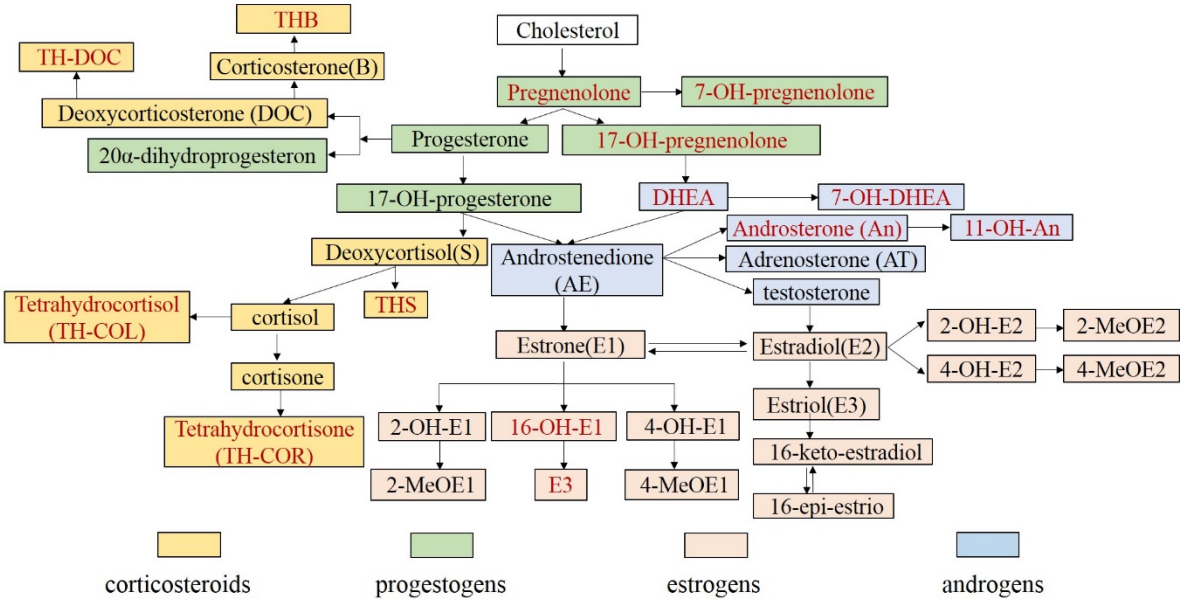


Figure 1. Metabolic pathways of steroids. Arrows represent ezymetic reactions between precursor and product steroids. The steroids in red represent the 3-OH steroids.

The three position of a steroid skeleton contains either a keto group or a hydroxyl group. Progesterone (P5), cortisol (COL), testosterone (TE) and some other steroids that are reproduced via the main biosynthesis routes contain a 3-keto group while pregnenolone, dehydroepiandrosterone and some other steroids contain a 3-OH group. The 3-OH steroids play important roles in the brain. Tetrahydrocortisol (TH-COL), tetrahydrocortisone (TH-COR), 3β,5α-tetrahydrodeoxycorticosterone (TH-DOC), Tetrahydrodeoxycortisol (THS) and tetrahydrocorticosterone (THB) are tetra-hydrocorticosteroids that are associated with stress [6, 7]. Pregnenolone, dehydroepiandrosterone, androsterone and their hydroxylated derivatives, such as 17α-hydroxypregnenolone (17-OH-P5), 7α-Hydroxydehydroepiandrosterone (7-OH-DHEA) and 11-β-hydroxyandrosterone (11-OH-An), are classified as neurosteroids because of their relevance to the modulation of various receptors such as the GABA receptor [3, 5, 8, 9]. The structures of the steroids were shown in Figure 2.

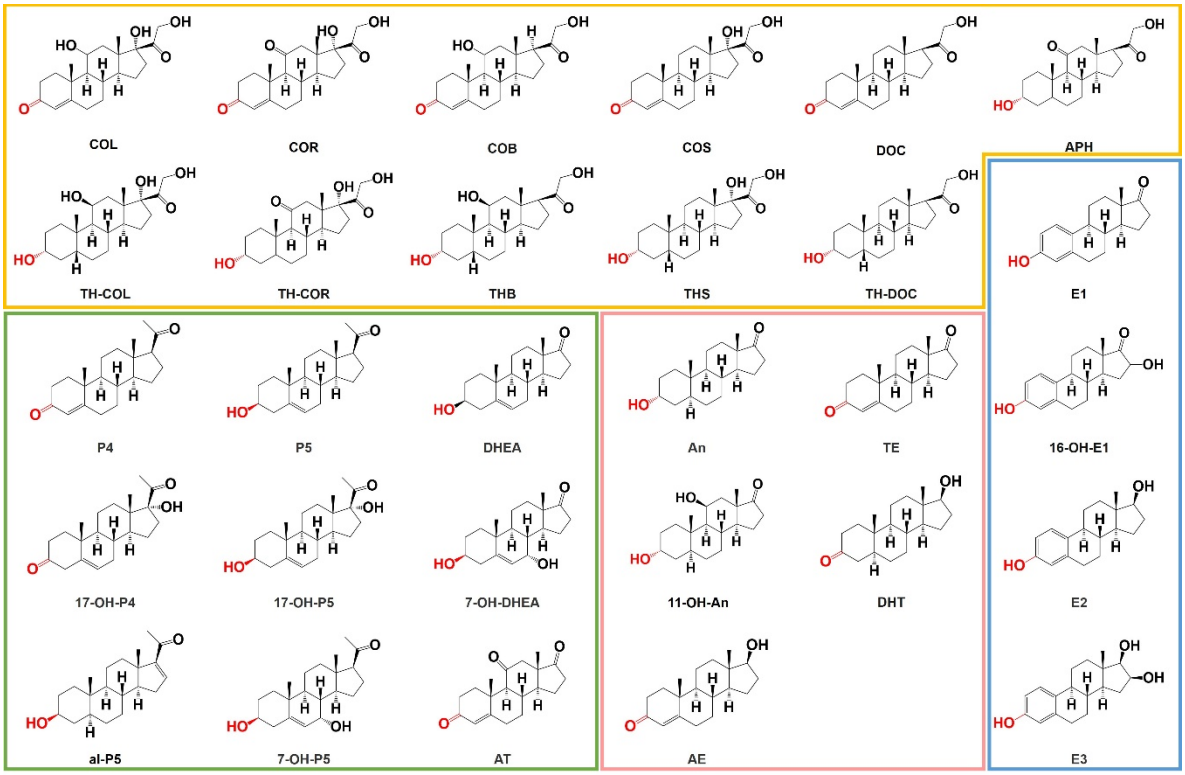


Figure 2. Structures of the steroids. The boxes shown in yellow, pink, green, and blue represent different classes of steroid hormones, i.e. corticosteroids, estrogens, progestogens, and androgens, respectively. Abbreviations: 11-OH-An, 11-β-hydroxyandrosterone 16-OH-E1, 16-hydroxyestrone 17-OH-P5, 17α-hydroxypregnenolone 17-OH-P4, 17α-Hydroxyprogesterone 7-OH-DHEA, 7α-hydroxydehydroepiandrosterone 7-OH-P5, 7α-hydroxypregnenolone Androstenedione, AE al-P5, allopregnenolone An, androsterone APD, alphadolone AT, Adrenosterone COB, Corticosterone COL, Cortisol COR, cortisone COS, 11-Deoxycortisol DHEA, dehydroepiandrosterone DHT, dihydrotestosterone DOC, 11-Deoxycorticosterone E1, Estrone E2, Estradiol E3, estriol P4, Progesterone P5, pregnenolone TE, Testosterone THB, tetrahydrocorticosterone TH-COL, tetrahydrocortisol TH-COR, tetrahydrocortisone TH-DOC, 3β,5α-tetrahydrodeoxycorticosterone THS, tetrahydrodeoxycortisol.

Profiling of steroids indicates the physiological state in endocrine systems, therefore it has been widely employed in the diagnoses and treatments of diseases [10]. The cascade-like pathway of steroids could also reveal the level change related to physiological activities. To develop a highly sensitive and accurate method for the determination of steroid levels is of great importance.

2. Detection methods for steroids

Many methods for detection of the steroids in biosample have been developed in the past several decades, including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) [11], gas chromatography (GC) [12-17], and liquid chromatography-mass spectrometry (LC-MS) [18-21]. RIA and ELISA are low-cost methods and could be conducted outdoor. However, one kit is only designed for one particular target, limiting the usage of multi-target detection in one sample. GC-MS allows multi-class profiling within one injection, but the non-volatile property of the steroids requires chemical derivatization. Direct detection of steroids could be achieved by LC-MS, but the 3-OH steroids are easily dehydrated in ion source when measured in the positive-ion mode, thus lowing the

detection sensitivity. Each method has both advantages and disadvantages, as described briefly in the following sections.

2.1 RIA and ELISA

RIAs and ELISAs are rapid, simple, and low-cost methods for quantitative analysis of steroids [11]. The kits are commercially available and the machines are relatively cheap and the analysis could be done in field studies [22, 23]. They are also popular in clinical diagnosis for their high sensitivity (pg/mL) [24, 25]. Immunoglobulin-based method is to bind the hormone and amplify the signal through an enzymatic reaction or radioactive signal. However, antibody cross-reactivity, matrix interferences and poor reproducibility limit the usage of these methods. Further, RIAs and ELISAs only allow the measurement of a single hormone per assay, thus limiting the ability to measure multiple hormones per sample.

2.2 GC-MS

Gas chromatography (GC) has excellent chromatographic resolution and multi-class profiling ability in metabolomics. However, steroids are non-volatile compounds and must be derivatized prior to the analysis. The hydroxyl groups were usually derivatized with the trimethylsilyl (TMS) derivatizing reagents or fluoro anhydride reagents [12-17].

To silylate steroids, various TMS derivatizing reagents are used, like N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) and N-O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) [12-16]. Steroids, which contain more than one hydroxyl group, might produce multiple derivatives, thus reducing the sensitivity and selectivity. The catalyst is recommended to increase the reaction efficiency, where a combination of trimethylchlorosilane, ammonium iodide and dithioerythritol is one of the commonly used reagents (Fig. 3).

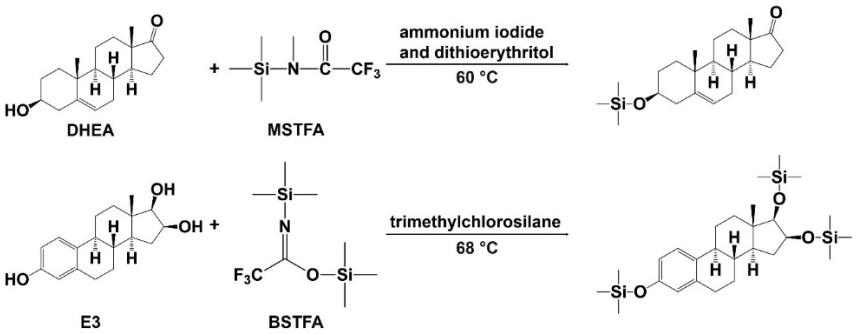


Figure 3. Derivatization of DHEA with MSTFA and E3 with BSTFA [12-16].

Estrogens and progesterone were derivatized by heptafluorobutyric anhydride (Fig. 4). The experiment was conducted by adding heptafluorobutyric anhydride–acetonitrile mixture (1:5, v/v) in the extract. The system was evaporated to dryness under a gentle stream of nitrogen to remove the solvent afterwards [17].

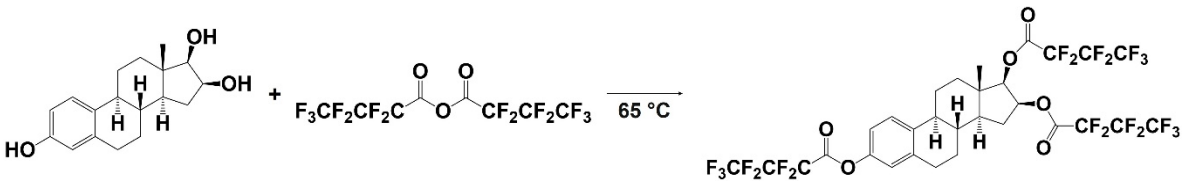


Figure 4. Derivatization of E3 with heptafluorobutyric anhydride [17].

Reproducibility is one of the issues in GC-MS-based method due to incomplete derivatization of multi-hydroxyl steroids.

2.3 LC-MS

Because of its high sensitivity, selectivity and multi-analyte capability, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become a popular method for the analysis of steroids in biosamples [18-21]. Electrospray ionization (ESI) is one of the most widely used soft ionization techniques in the analysis of low molecular weight targets. The tandem mass spectrometry (MS-MS) improves the selectivity, and especially, is powerful for the case that all the components cannot be separated from one another in the LC.

2.3.1 Direct detection by LC-MS

Most of the steroids contain carbonyl groups and be protonated in the ESI. On the three position of cholesterol skeleton, there is either a keto group or a hydroxyl group. Compared with steroids containing a 3-keto group, the structures of which are maintained during ionization, 3-OH steroids are easily dehydrated when measurements are made in the positive-ion mode [26, 27].

In this case, the precursor ions of 3-OH steroids, used in the multiple reaction monitoring (MRM) measurement mode (see Experimental section), are $[M+H-H_2O]^+$ or $[M+H-2H_2O]^+$ rather than $[M+H]^+$ (Table 1) [19, 20, 28-31]. However, the formation of multiple dehydration products in the ion source eventually decreases the sensitivity of detection of the precursor ion to be subjected to MRM. The lack of product ions derived from the backbone might also result in an ambiguous structural characterization.

Table 1. Parameters for steroid analysis using liquid chromatography tandem mass spectrometry [20].

Steroid Common Name	Abbreviations	Absolute Mass (*m/z*)	Ion/Reaction	Precursor Ion (m/z)	Product Ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)	Retention Time (min)
Dehydroepiandrosterone	DHEA	288.4	$[M+H-2H_2O]^+$	253.3	197.3	75	10	25	10	11.21
Estradiol	E_2	272.4	$[M+H-H_2O]^+$	255.3	159.2	75	10	25	10	10.91
Estradiol-$^{13}C_3$	E_2-$^{13}C_3$	275.4	$[M+H-H_2O]^+$	258.2	162.2	50	10	35	10	10.91
Estriol	E_3	288.4	$[M+H-H_2O]^+$	271.3	133.4	75	10	25	10	10.01
17-Hydroxypregnenolone	17-OH-P_5	332.5	$[M+H-H_2O]^+$	315.4	297.3	40	5	20	5	10.97
Pregnenolone	P_5	316.5	$[M+H-H_2O]^+$	299.4	161.4	75	15	25	10	13.37
Pregnenolone-$^{13}C_2$-d_2	P_5-$^{13}C_2$-d_2	320.5	$[M+H-H_2O]^+$	303.6	161.3	40	5	40	10	13.36

Sodium ion, which has a similar affinity for proton and is often observed as the adduct ion, also has an affect on the reproducibility of quantitation in MRM. This dehydration phenomenon was essentially eliminated when 3-OH steroids are measured as deprotonated ions in negative-ion mode. In this case, as shown in Table 2, estradiol and estriol were measured as $[M-H]^-$ as the precursor ion [29].

Table 2. Signal to noise (S/N) ratios of MRM transitions of steroids (c=1 ng/mL) in negative ion modes [29].

Steroid	Species	MRM
β-Estradiol	$[M-H]^-$	271/145
Estriol	$[M-H]^-$	287/171

However, 3-OH steroids that are amenable to the measurement in the negative mode have been less than ten kinds, rendering overall analysis by LC-MS/MS limited.

2.3.2 Chemical derivatization for LC-MS

Owing to the lack of acidic or basic groups in most of steroids, they are deficient in ionization, making the LC-MS method difficult. The introduction of an amino group by chemical derivatization could dramatically enhance the detection sensitivity. Meanwhile, chemical derivatization is also useful for avoiding dehydration when the ketone or hydroxyl group could be blocked by the derivatization.

The Girard-P reagent is a widely used derivatization reagent for the detection of ketolic metabolites by LC-MS [21, 32]. The carbazinamide reacts with the carbonyl group and form the semicarbazone under acid condition (Figure 5). In MS/MS analysis, the characteristic peak at m/z 80 was a protonated pyridine derived from Girard-P reagent.

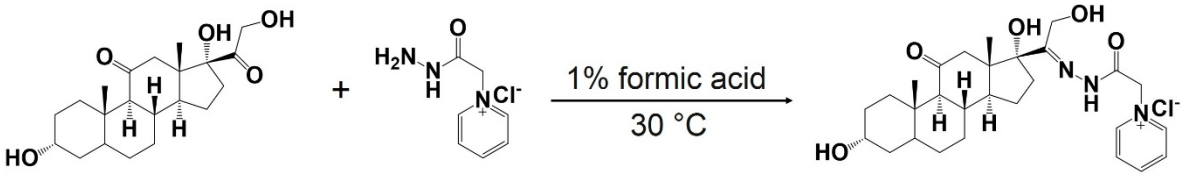


Figure 5. Derivatization of TH-COR with Girard-P reagent [21].

Hydroxylamine has also been used for the derivatization of ketolic steroids (Figure 6) [33, 34]. After derivatization, the oxime conjugate was the major peak in the MS spectrum for 17-OH-P5 and DHEA.

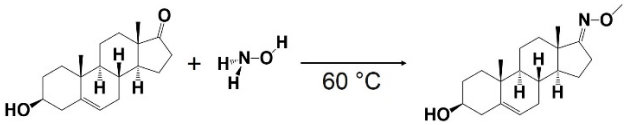


Figure 6. Derivatization of DHEA with hydroxylamine [33] .

The phenylhydrazine, 2-nitro-4-trifluoromethylphenyl hydrazine (NFPH) was used for labeling the keto group of THB. The acyl chloride, dinitrobezoyl esters (DNBC) was applied for derivatization of

both keto and hydroxyl groups. (Figure 7) By taking this strategy, the THB derivatives could be detected by atmospheric pressure chemical ionization (APCI) in negative mode.

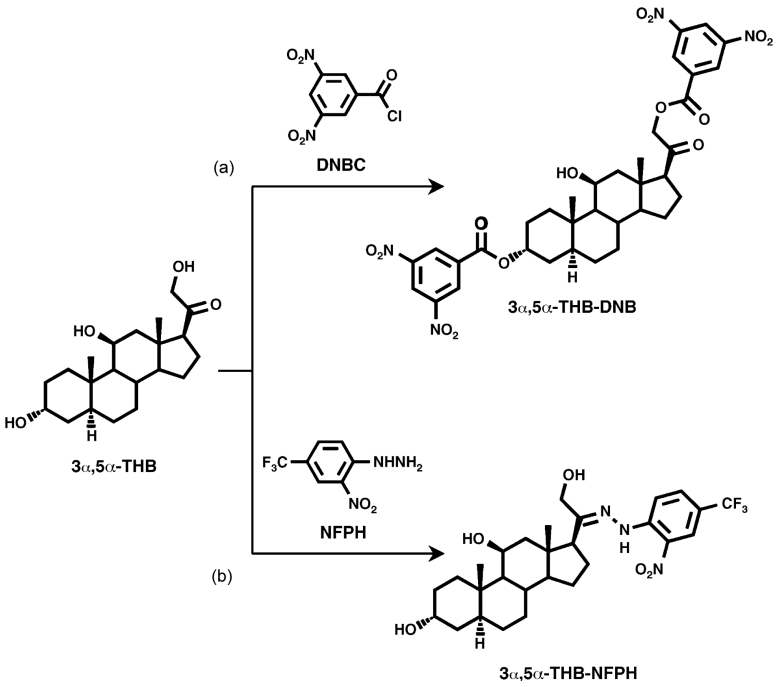


Figure 7. Derivatization of 3α, 5α-THB with (a) DNBC and (b) NFPH [35].

The THB-DNB derivative provided its deprotonated ion as precursor ion at m/z 738.4 in MRM analysis. However, the derivative was relatively stable, and the intensity of the product ions was very low. NFPH reacted with the carbonyl group at the 20-position of the THB, and the derivative gave its deprotonated ion ($[M-H]^-$, m/z 552.4) and dehydrated ion ($[M-H_2O]^-$, m/z 535.6) as the major ions in the APCI-MS in the negative-ion mode. In these analyses, neither derivatization strategies could offer a better result.

Dansyl chloride is a well-known phenolic and amino group labeling reagent and was often applied for analysis of estrogens (Fig. 8) [20, 21, 32]. The derivatives could give an intense MS/MS peak at m/z 171, which corresponds to the 5-dimethylamino-naphthalene group derived from the skeleton of dansyl chloride (Fig.9) [36].

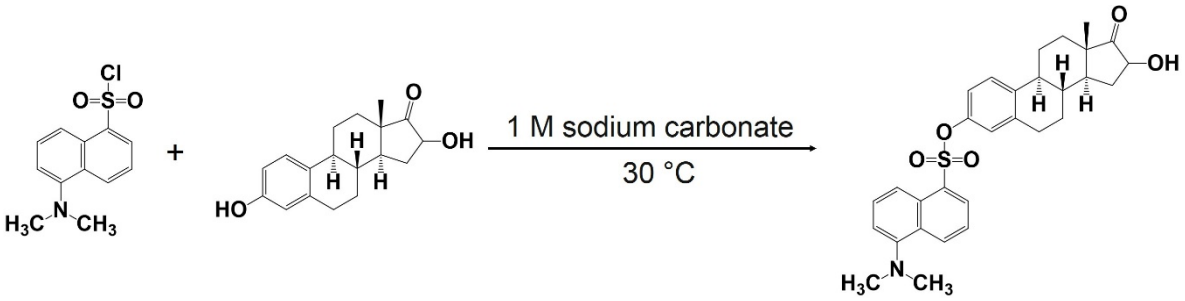


Figure 8. Derivatization of 16-OH-E1 with dansyl chloride [32].

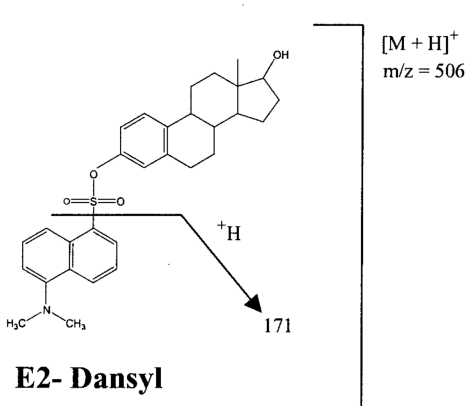


Figure 9. Fragmentation behavior of the dansyl chloride derivatized E2 [36].

Despite of the instability and the poor fragmentation efficiency of the products for some reagents, chemical derivatization remains the most powerful tool to enhance the detection sensitivity in steroid analysis by LC-MS/MS nowadays. However, it requires additional incubation, purification or extraction steps.[37]

Since the detection of steroids in biosample is of great importance, I would like to establish a fast and sensitive method which allows for profiling multi-class steroids. Among the detection strategies mentioned above, LC-MS is the most powerful method for its high sensitivity and is used as an analytical tool in this study. Since the dehydration phenomenon of 3-OH steroids remains to be overcome, I developed a method for the quantitation of 3-OH steroids by LC-MS/MS. Since lithium ion has a higher affinity for the carbonyl group, compared with protons, ammonium and sodium ions, it was possible to detect 3-OH steroids that contain keto groups solely as the lithiated forms, which leads to an enhancement in their ion intensities.

In Chapter I, I described the novel method utilizing Li^+ ion as an adduct ion and showed its performance using standard 3-OH steroids. In Chapter II, I described the optimization of sample pretreatment procedure for isolating free steroids from mouse brain tissue, and tried to analyze 3-OH steroids using Li ion adduction method (Li-method). In Chapter III, in order to fulfil the overall quantitation of the endogenous steroids in mouse brain, the normal proton adduction method (H-method) was applied to the mouse brain and compared with the Li-method. As the results with both methods, thirteen steroids could be identified from one mouse brain tissue sample, among which 16-hydroxyestrone, tetrahydrocorticosterone, and 17α-hydroxypregnenolone were, for the first time, identified in the mouse brain.

Chapter I. A method utilizing Li^+ ion as an adduct ion and its performance using standard 3-OH steroids

I.1 Introduction

Additives are used in LC mobile phases to improve the sensitivity in MS. Chemical properties of the additive have a significant effect on analyte response and stability in ESI[38]. Polar neutral compounds that cannot be easily ionized by protonation or deprotonation (esters, carbohydrates and many kinds of lipids) might be ionized via adduct ion formation, like ammonium, sodium, lithium ions or silver ions in positive ion mode ($[M+NH_4]^+$, $[M+Na]^+$, $[M+Li]^+$) [39-43]. These adducts could be formed by adding the additives to the mobile phase or dissolving the salts in sheath liquid which will be mixed with the eluent after the column.

I.1.1 Ammonium adducts as precursor ions in LC-MS analysis

$[M+NH_4]^+$ was used for the detection of P5 and DHEA when the ESI ionization were operated at room temperature (Table I.1) [44]. In this case, since water-based solvent system was hard to vaporize in room temperature at atmospheric pressure, organic solvent could be a choice for the mobile phase, the separation of analytes was limited.

Table I.1. Relative Retention Times and MRM transitions of DHEA, estradiol (E), tetrahydrodeoxycorticosterone (THD) and PREG (pregnenolone) [44].

compound	relative retention time (min)	SRM pair	precursor ion	product ion
DHEA	0.84	306→271	$[M + NH_4]^+$	$[M + H - H_2O]^+$
		306→253	$[M + NH_4]^+$	$[M + H - 2H_2O]^+$
E	0.84	290→273	$[M + NH_4]^+$	$[M + H]^+$
		290→273	$[M + NH_4]^+$	unknown
THD	0.88	352→335	$[M + NH_4]^+$	$[M + H]^+$
		352→317	$[M + NH_4]^+$	$[M + H - H_2O]^+$
PREG	0.98	334→317	$[M + NH_4]^+$	$[M + H]^+$

I.1.2 Metal ion adducts to precursor ions in LC-MS analysis

The use of lithium or silver salts result in the formation of lithium or silver adducts instead of the protonated ion, while sodium adducts $[M+Na]^+$ are often formed in addition to $[M+H]^+$ ions since sodium ions always exist in the mobile phase due to impurities derived from sample vials and LC-lines, and may decrease the sensitivity of the analysis owing to the ion suppression.

I.1.2.1 Silver ion adduct for detecting small molecules

Silver ions have also been used to form adducts of 11-OH-An, DHEA E2 and E3 [45]. Howeversince Ag is made up of two stable isotopes, ^{107}Ag (51.839%) and ^{109}Ag (48.161%), all possible silverated adducts are observed at approximately half intensity. Moreover, , as shown in Table I.2, Ag^+, which resulted from the neutral loss of the steroid molecule, and $[M+Ag-2H_2O]^+$ did not give information on the structure of the steroid to some extent. Silver ion could have high affinity with sulfur compounds, which is not suitable for the analysis of biosamples in general.

Table I.2. MRM transitions and retention times for the screening analysis of 84 anabolic steroids [45].

Name	Abbreviation	M.W	Q1 (m/z)	Q3 (m/z)
11β-OH-Androsterone	11β-OH-A	306	413	377
Dehydroepiandrosterone	DHEA	288	395	107
17β-Estradiol	E2	272	381	157
Estriol	E3	288	395	287

I.1.2.2 Lithium ion adduct for detecting small molecules

Lithium ions are able of forming cationic adducts with a variety of compounds [38, 46]. Adams and Gross have used the lithium ion to improve the fragmentation of lipids and the determination of the double bond location in 1980s [47, 48]. A post-column addition method, using 0.25 mM lithium chloride, was applied for analysis of ouabain in human serum (Fig. I.1).[49, 50]

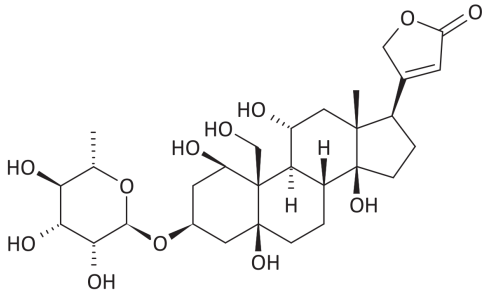
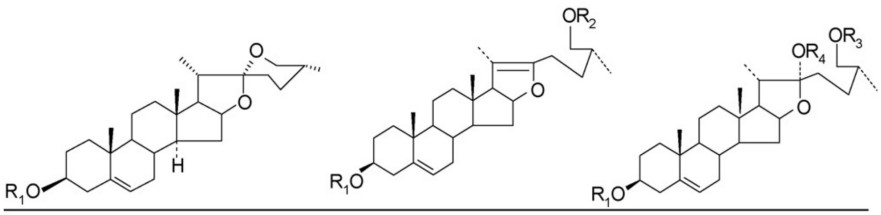


Figure I.1. Chemical structure of ouabain.

CID (collision induced dissociation) mass spectra of the lithium adducted pseudoprotodioscin and methyl protodioscin were obtained using ESI-MS^n in Fig. I.2 [51].



Compounds	R_1	R_2	R_3	R_4	MW
dioscin	Rha(1 →4)[Rha(1 →2)]-Glc				868
pseudoprotodioscin	Rha(1 →4)[Rha(1 →2)]-Glc	Glc			1030
protodioscin	Rha(1 →4)[Rha(1 →2)]-Glc		Glc	H	1048
methyl protodioscin	Rha(1 →4)[Rha(1 →2)]-Glc		Glc	CH_3	1062

Glc =β-D-glucopyranosyl, Rha =α-L-rhamnopyranosyl

Figure **I.2.** Structures of dioscin, pseodoprotodiscin, protodioscin and methyl protodioscin.

1α,25-dihydroxyvitamin D having a structure similar to a steroid was determined in lithiated form by adding lithium acetate to the mobile phase (Fig. I.3) [39, 52]. MRM was set at 423.3 → 369.3 for 1,25-$(OH)_2$vitamin D , which is a triply dehydrated ion.

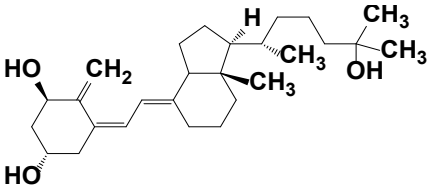


Figure I.3. Structure of 1α,25-dihydroxyvitamin D.

Using a similar strategy, steroid glycoside conjugates were quantified in porcine plasma samples (Fig. I.4) [53].

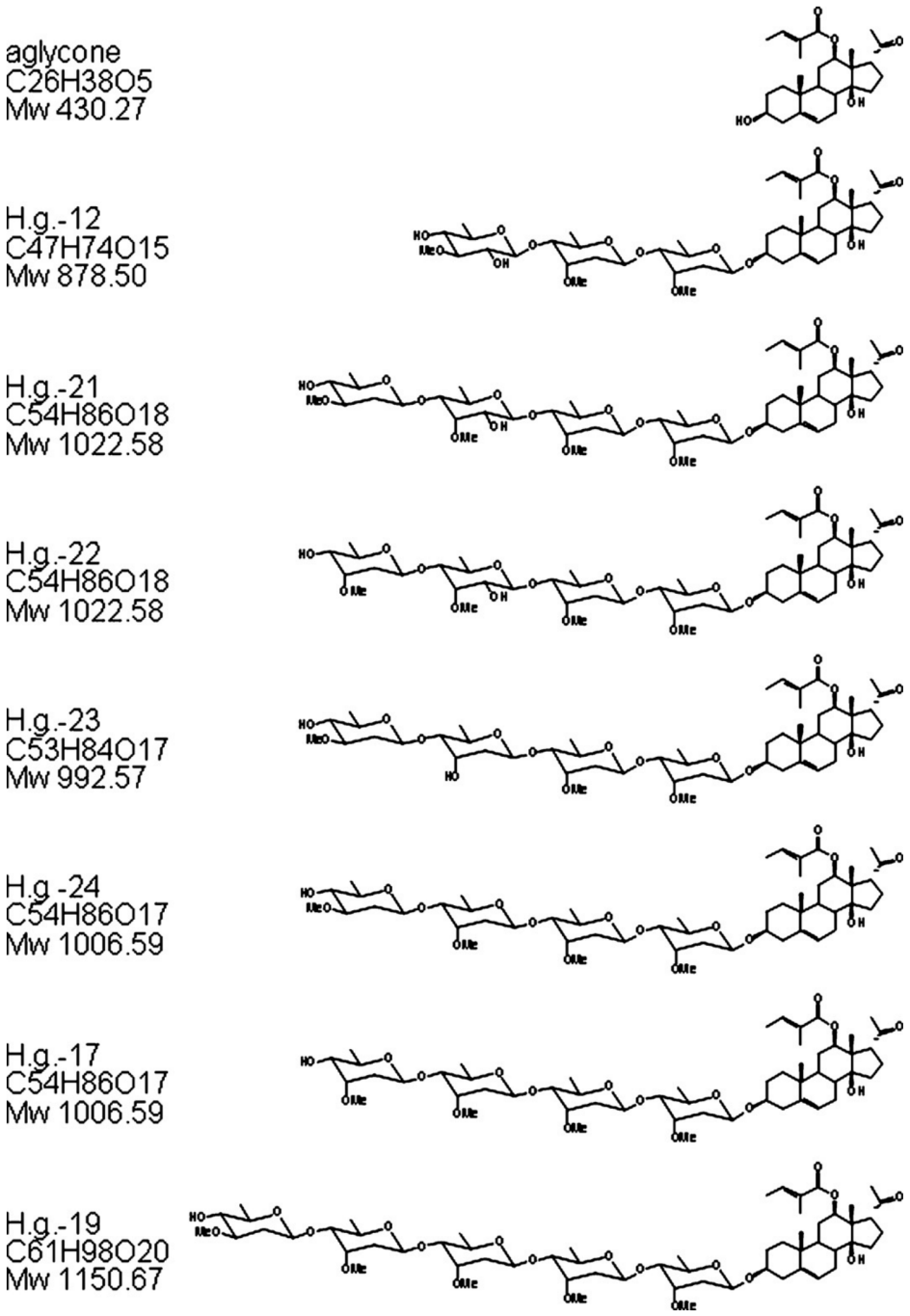


Figure I.4. Structures of the main Hoodia gordonii (H.g.) steroid glycosides. The H.g. molecules consist of a steroid core with a glycosidic chain esterified to tiglic acid (2-methylbut-2-enoic acid) [53].

These cases prompted Sesek-Rahkonen to develop a similar method for the analysis of estradiol in human serum and tissue. However, in this case, a lithium adduct was not efficiently formed [54], which could be explained by the fact that estradiol contains only two hydroxyl groups, thus lowering the affinity of this compound for Li ion, compared to 3-OH steroids with multiple hydroxyl and/or keto groups.

In 2013, Bao et al. used 25 mM lithium acetate in the mobile phase to form the lithium adduct of 20(S)-protopanaxadiol [55]. Since 20(S)-protopanaxadiol, which does not contain a keto group (see Fig. I.5), showed a moderate affinity for lithium, a relatively higher concentration (25 mM) of lithium acetate was used. Moreover, the setting of the MRM transition ($[M+Li]^+ \rightarrow [M+Li-H_2O]^+$) seemed unreliable since many natural metabolites also produce dehydrated ions upon CID.

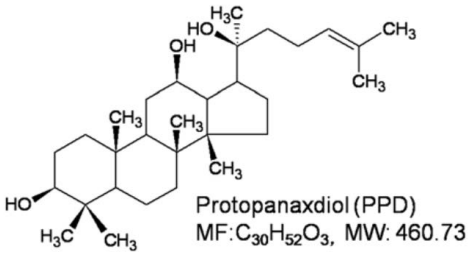


Figure I.5. Chemical structure of 20(S)-protopanaxadiol [55].

The above compounds have a steroid skeleton, and all contain a 3-OH or a 3-OR group. The free steroid hormones, as reported above, have never been quantified in the form of their lithium adducts by LC-MS/MS.

I.2 Material and Methods

I.2.1 Chemicals

HPLC grade methanol (MeOH), acetonitrile (ACN) and ethyl acetate (EA), 99.998% trace metals basis lithium chloride (LiCl), 99.95% trace metals basis lithium acetate (LiOAc), pregnenolone (P5), 17α-hydroxypregnenolone (17-OH-P5), dehydroepiandrosterone (DHEA), estriol (E3),

dehydroepiandrosterone-2,2,3,4,4-d_5 (DHEA-d_5), and pregnenolone-20,21-$^{13}C_2$-16,16-d_2 (P5-$^{13}C_2$,d_2) were purchased from Sigma-Aldrich (Tokyo, Japan). Alphadolone (APD), allopregnenolone (al-Preg), tetrahydrocortisol (TH-COL), tetrahydrocortisone (TH-COR), 11-β-hydroxyandrosterone (11-OH-An), tetrahydrocorticosterone (THB), 3β,5α-tetrahydrodeoxycorticosterone (TH-DOC), tetrahydrodeoxycortisol (THS), 16-hydroxyestrone (16-OH-E1), 16-keto-17β-estradiol-2,4,6,6,9-d_5 (16-keto-E2-d_5) and tetrahydrocortisone-2,2,4,4,21,21-d_6 (TH-COR-d_6) were supplied by Toronto Research Chemicals (North York, Canada). Androsterone (An) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 7α-hydroxydehydroepiandrosterone (7-OH-DHEA) and 7α-hydroxypregnenolone (7-OH-P5) were supplied by Nacalai tesque (Kyoto, Japan). HPLC grade formic acid was obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Ultrapure water was prepared using a puric ω (Tokyo, Japan). The structures of the 3-OH steroids in this analysis are shown in Figure I.6.

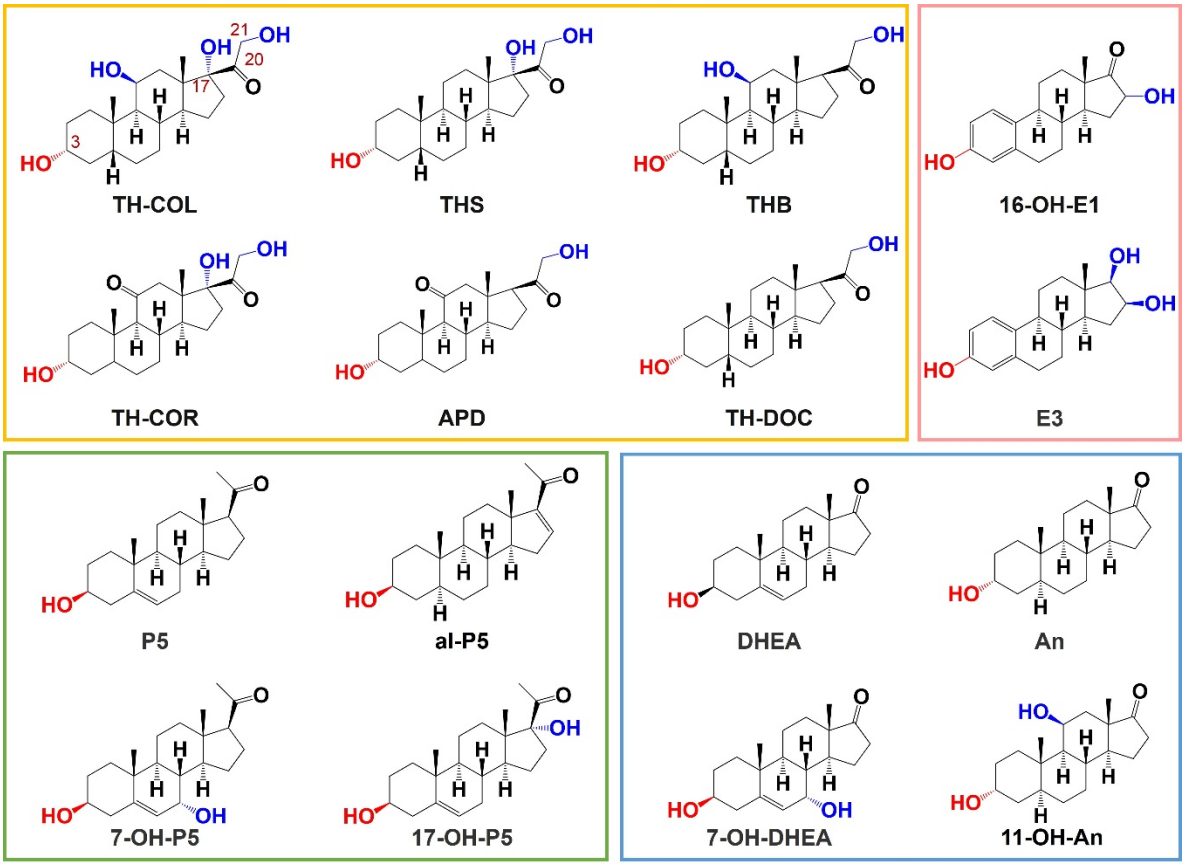


Figure I.6. Structures of the 3-OH steroids used in this analysis. The boxes shown in yellow, pink, green, and blue represent different classes of steroid hormones, i.e. corticosteroids, estrogens, progestogens, and androgens, respectively.

I.2.2 Preparation of standard stock, calibration, and quality control stock solutions

Each steroid standard was prepared in methanol at a concentration of 4 mg mL^{-1} as respective stock solutions and stored in -80 °C. These stock solutions were mixed and diluted to 10 ng μL^{-1} with 40% MeOH as mixed stock solutions at -80 °C. The working standard solutions were prepared at concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500 pg μL^{-1} with 40% MeOH. An internal standard (IS) mixture solution of 0.04 ng μL^{-1} 16-keto-E2-d_5 and DHEA- d_5, and 0.02 ng μL^{-1} of TH-COR-d_6 and P5-$^{13}C_2$,d_2 was prepared in 40% MeOH.

I.2.3 LC-MS/MS

The UPLC-MS/MS analysis was performed using an Agilent 1290 Infinity II and 6470 triple quadrupole mass spectrometer equipped with an ESI ion source (Agilent Technologies, Inc. Santa Clara, CA). Chromatographic separation was achieved with an Agilent Eclipse Plus C18 RRHD 2.1x100 mm, 1.8 µm column, which was maintained at 40 °C. The mobile phase consisted of solvent A (0.1% formic acid in deionized water) and solvent B (0.1% formic acid in MeOH). The elution gradient was from 40-80.0 % B from 0 to 8 min, maintained at 80 % B from 8 to 10 min, 80-40.0 % from 10.0 to 10.10 min and held at 40 % B from 10.1 to 13.1 min. The injection volume was 10 µL. The MRM mode was applied for the detection and quantitation of all steroids with two transitions optimized for each targeted compound. The ESI source parameters were set as follows: the capillary voltage was -4500 V in the positive ion mode, the nebulizer (N_2) pressure was 55 psi, the drying gas (N_2) temperature and flow rate were 210 °C and 13 L min^{-1}, respectively, and sheath gas temperature was 275 °C. The post-column addition of 0.2 mM LiCl in H_2O was carried out with an Agilent 1100 binary pump as the auxiliary pump. The column effluent (0.4 mL min^{-1}) and auxiliary solution (0.4 mL min^{-1}) were mixed at the T-connector and passed through two in-line filters (Agilent 1290 inline filter, 0.3µm), which were connected in tandem, prior to reaching to the ion source. The MRM parameters for 3-OH steroids are summarized in Table I.3 and Table I.4. Note that since Li ion salts such as LiCl and LiOAc are non-volatile, the amount of LiCl introduced to the ion source was reduced

to 0.2 mM, which was sufficient to form complete Li adducts of the 3-OH steroids (see Fig. I.7), and the absolute amount of LiCl introduced into MS was reduced to 0.48 μmol (20.3 μg)/analysis by controlling the two-way switching valve to either MS (6 min for the analysis) or waste (7 min for the column equilibration) by an Agilent MassHunter Acquisition system. A lithium adduct of 20(S)-protopanaxadiol was observed with 25 mM lithium acetate in a previous report [55], which might cause ionization to be suppressed or plug the capillary inlet.

To compare the lithium adduct method (Li method) with the previous protonated method (H method), ESI source parameters were set similarly in both methods, which were slightly different from the above: the nebulizer (N_2) pressure was set at 30 psi and the sheath gas temp was 375 ^oC to enhance the dehydration of steroids. The auxiliary pump was off when conducting the "H method". The MRM parameters for the "H method" are summarized in Table I.4.

Table I.3. Parameters for the analysis of 3-OH steroids by LC-MS/MS. The LiCl was introduced from the auxiliary pump.

Analyte	Precursor ion form	RT/ min	Precursor Ion m/z	Fragmentor	Product Ion m/z	Collision Energy	Expected fragment formula	IS
E3	$[M+Li]^+$	3.13	295.2	165	**277.2**	**24**	**$[M+Li-H_2O]^+$**	16-keto-E2-d_5
					233.1	24	$[M+Li-C_2H_6O_2]^+$	
16-keto-E2-d_5	$[M+Li]^+$	3.53	298.2	170	**79.1**	**29**	**$[C_6H_3d_2]^+$**	ISTD
					223.2	29	$[M+Li-C_3H_5DO_2]^+$	
16-OH-E1	$[M+Li]^+$	3.73	293.2	170	**79.1**	**28**	**$[C_3H_4O_2Li]^+$**	16-keto-E2-d_5
					275.2	22	$[M+Li-H_2O]^+$	
7-OH-DHEA	$[M+Li]^+$	4.52	311.2	155	**275.2**	**20**	**$[M+Li-2H_2O]^+$**	16-keto-E2-d_5
					237.2	24	$[M+Li-C_4H_{10}O]^+$	
TH-COL	$[M+Li]^+$	5.61	373.3	210	**343.3**	**37**	**$[M+Li-CH_2O]^+$**	TH-COR-d_6
					325.3	39	$[M+Li-CH_2O-H_2O]^+$	
7-OH-P5	$[M+Li]^+$	5.84	339.2	155	**303.3**	**20**	**$[M+Li-2H_2O]^+$**	TH-COR-d_6
					265.2	24	$[M+Li-C_4H_{10}O]^+$	
TH-COR-d_6	$[M+Li]^+$	5.88	377.2	220	**345.3**	**33**	**$[M+Li-Cd_2O]^+$**	ISTD
					327.3	41	$[M+Li-Cd_2O-H_2O]^+$	
TH-COR	$[M+Li]^+$	5.89	371.2	210	**341.2**	**34**	**$[M+Li-CH_2O]^+$**	TH-COR-d_6
					323.2	39	$[M+Li-CH_2O-H_2O]^+$	
11-OH-An	$[M+Li]^+$	5.93	313.2	180	**293.2**	**35**	**$[M+Li-H_2O-H_2]^+$**	TH-COR-d_6
					275.2	32	$[M+Li-2H_2O-H_2]^+$	
THB	$[M+Li]^+$	6.12	357.2	190	**93**	**42**	**$[C_4H_6O_2Li]^+$**	TH-COR-d_6
					107.1	45	$[C_5H_8O_2Li]^+$	

APD	$[M+Li]^+$	6.17	355.2	195	**337.2**	**35**	**$[M+Li-H_2O]^+$**	TH-COR-d_6
					295.2	35	$[M+Li-C_2H_4O_2]^+$	
DHEA-d_5	$[M+Li]^+$	6.72	300.2	155	**281.2**	**22**	**$[M+Li-HdO]^+$**	ISTD
					107.1	53	$[C_8H_7d_2]^+$	
DHEA	$[M+Li]^+$	6.76	295.2	155	**277.2**	**22**	**$[M+Li-H_2O]^+$**	DHEA-d_5
					105.1	53	$[C_8H_9]^+$	
17-OH–P5	$[M+Li]^+$	6.87	339.2	200	**93.1**	**45**	**$[C_7H_9]^+$**	DHEA-d_5
					81.1	55	$[C_6H_9]^+$	
TH-DOC	$[M+Li]^+$	7.11	341.2	180	**93**	**38**	**$[C_4H_6O_2Li]^+$**	TH-COR-d_6
					107	38	$[C_5H_8O_2Li]^+$	
THS	$[M+Li]^+$	7.79	357.2	190	**327.2**	**37**	**$[M+Li-H_2O]^+$**	TH-COR-d_6
					309.3	42	$[M+Li-CH_2O-H_2O]^+$	
An	$[M+Li]^+$	8.24	297.2	180	**279.2**	**28**	**$[M+Li-H_2O]^+$**	P5-$^{13}C_2$,d_2
					155.1	51	$[C_{10}H_{12}OLi]^+$	
P5-$^{13}C_2$,d_2	$[M+Li]^+$	8.51	327.2	170	**309.3**	**25**	**$[M+Li-H_2O]^+$**	ISTD
					161.1	33	$[C_{12}H_{17}]^+$	
P5	$[M+Li]^+$	8.53	323.2	170	**43.1**	**55**	**$[CH_3CO]^+$**	P5-$^{13}C_2$,d_2
					105.1	55	$[C_8H_9]^+$	
al-P5	$[M+Li]^+$	8.70	323.2	190	**305.2**	**29**	**$[M+Li-H_2O]^+$**	P5-$^{13}C_2$,d_2
					43.1	53	$[CH_3CO]^+$	

The m/z values of product ions (MRM transitions) for each analyte in bold was used as the quantifying ion all other ions were used as qualifying ions. ISTD, internal standard

Table I.4. Parameters for the analysis of 3-OH steroids by LC-MS/MS. The auxiliary pump was off.

Analyte	Precursor ion form	RT/ min	Precursor Ion m/z	Fragmentor	Product Ion m/z	Collision Energy
E3	$[M-H_2O+H]^+$	3.13	271.2	110	**133.1**	**25**
					253.2	9
16-keto-E1-d_5+H	$[M+H]^+$	3.53	292.2	90	**274.2**	**13**
					203.2	25
16-OH-E1	$[M+H]^+$	3.73	287.2	110	**251.1**	**13**
					199.1	17
7-OH-DHEA	$[M-2H_2O+H]^+$	4.52	269.2	140	**91.1**	**55**
					81.1	29
TH-COL	$[M-2H_2O+H]^+$	5.61	331.3	130	**301.2**	**13**
					105.1	55
7-OH-P5	$[M-2H_2O+H]^+$	5.84	297.2	110	**81.1**	**29**
					145.1	25
TH-COR-d_6-H2O	$[M-H_2O+H]^+$	5.88	353.2	140	**335.3**	**17**
					153.1	25
TH-COR	$[M+H]^+$	5.89	365.2	100	**149.1**	**25**
					121.1	33
11-OH-An	$[M-2H_2O+H]^+$	5.93	271.2	120	**105.2**	**49**
					91	55
THB	$[M-2H_2O+H]^+$	6.12	315.2	140	**91.1**	**55**
					105	55
APD	$[M+H]^+$	6.17	349.2	110	**295.2**	**13**
					105	55
DHEA	$[M-H_2O+H]^+$	6.76	271.2	130	**197.1**	**21**
					213.2	17
17-OH-P5	$[M-H_2O+H]^+$	6.87	315.2	90	**81.1**	**37**
					105.1	53
TH-DOC	$[M-H_2O+H]^+$	7.11	317.2	120	**281.2**	**13**
					105	53
THS	$[M-2H_2O+H]^+$	7.79	315.2	110	**279.2**	**13**
					105.1	49

An	$[M-H_2O+H]^+$	8.24	273.2	140	**255.2**	**13**
					161.1	21
P5-$^{13}C_2d_2$	$[M-H_2O+H]^+$	8.51	303.2	130	**285.3**	**9**
					45	55
P5	$[M-H_2O+H]^+$	8.53	299.2	120	**159.1**	**25**
					43.1	55
al-P5	$[M+H]^+$	8.70	317.2	130	**281.2**	**18**
					43.1	15

The m/z values of product ions (MRM transitions) for each analyte in bold was used as the quantifying ion all other ions were used as qualifying ions.

I.2.4 Data processing

Data were acquired using an Agilent MassHunter Acquisition system, and processed using Agilent MassHunter Quantitive Analysis, Microsoft Excel, and OriginLab 2018 (Academic).

I.3 Results and discussion

I.3.1 Mass spectrometry

The mass spectrometric and tandem mass spectrometric behavior of the lithiated steroids were examined in the positive-ion ESI mode by direct infusion. As shown in Fig. I.7, when the mobile phase was aqueous 0.1% FA and MeOH, numerous signals for dehydrated ions and sodium adducts were observed in the mass spectra. Since TH-COR contains three hydroxyl groups, multi-dehydrated ions were observed, as shown in Fig. I.7b-3. This phenomenon was also observed for other steroids, such as 7-OH-DHEA (Fig. I.7b-2), 11-OH-An (Fig. I.7b-4), and, THB (Fig. I.7b-5) and 17-OH-P5 (Fig. I.7b-6), both of which contain two hydroxyl groups. The sodium adduct peaks were very strong for 16-OH-E1 (Fig. I.7b-1), 11-OH-An, THB, and 17-OH-P5. When the mobile phase was aqueous 0.2 mM LiCl and MeOH, the lithium adducts became the dominant peaks in the spectra (Fig. I.7a). Li^+, which acts as a Lewis acid, has a stronger affinity for the lone

pair electrons of the hydroxyl or carbonyl groups on the steroids compared with other alkaline metal ions.

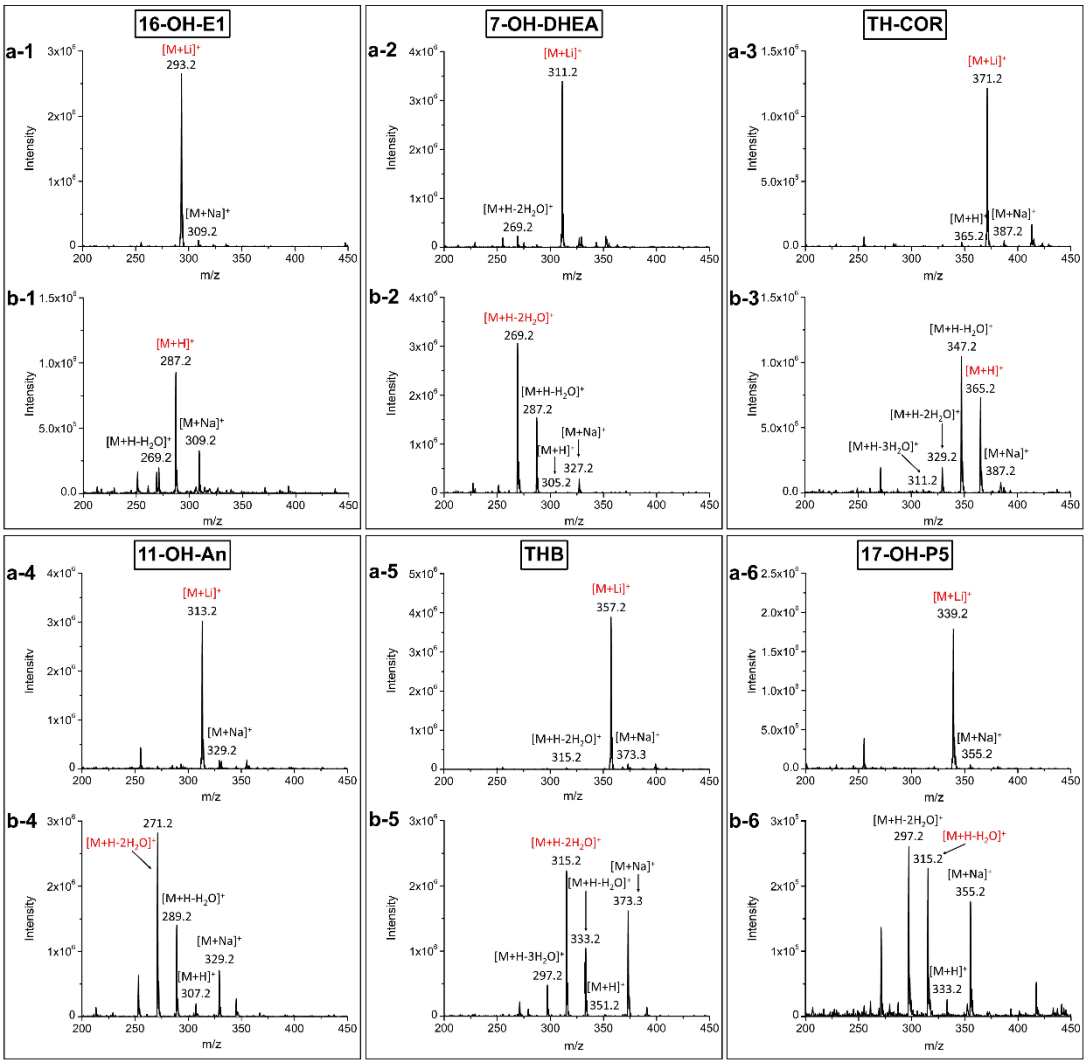


Figure I.7. ESI-MS spectra of the 3-OH steroids. The mobile phase consisted of: solvent A: 0.2 mM LiCl in deionized water (a) and 0.1% FA in deionized water (b) and solvent B (MeOH). 1:16-OH-E1, 2: 7-OH-DHEA, 3: TH-COR, 4: 11-OH-An, 5: THB, 6: 17-OH-P5. 200 ng of each steroid, in 20% of solvent B, was introduced directly to the MS through a two-way valve. The red ions represent the precursor ions used for the MRM analysis.

I.3.2 MS/MS fragmentation

Product ions for lithiated steroids were mainly derived from dehydration that occurred at the hydroxyl groups or by ring-cleavage (Fig. I.8) and were clearly observed with a relatively high collision energy (more than 20 eV) (Table I.4). Since the lithium ions were preferably attached to

keto groups, the fragmentation behavior was simple and some specific fragments were produced (Fig. I.8a). Meanwhile, protonated steroids (dehydrated forms) showed complex fragmentation patterns upon CID (Fig. I.8b). Those fragments could be originated from the tetracyclic rings and were produced with a higher collision energy. In addition, since the proton was delocalized in the molecules, the complex fragmentation, which was mainly observed at a 14 Da interval, can be attributed to the cleavage in each carbon-carbon bond on the skeleton. The satellite peaks with ± 2 Da to the main peaks could be accounted for by the unsaturated double bonds in the structure. Owing to the complexity and dispersion of the fragment ions, it becomes difficult to define the MRM transition and to obtain sufficient intensities for the MRM transitions.

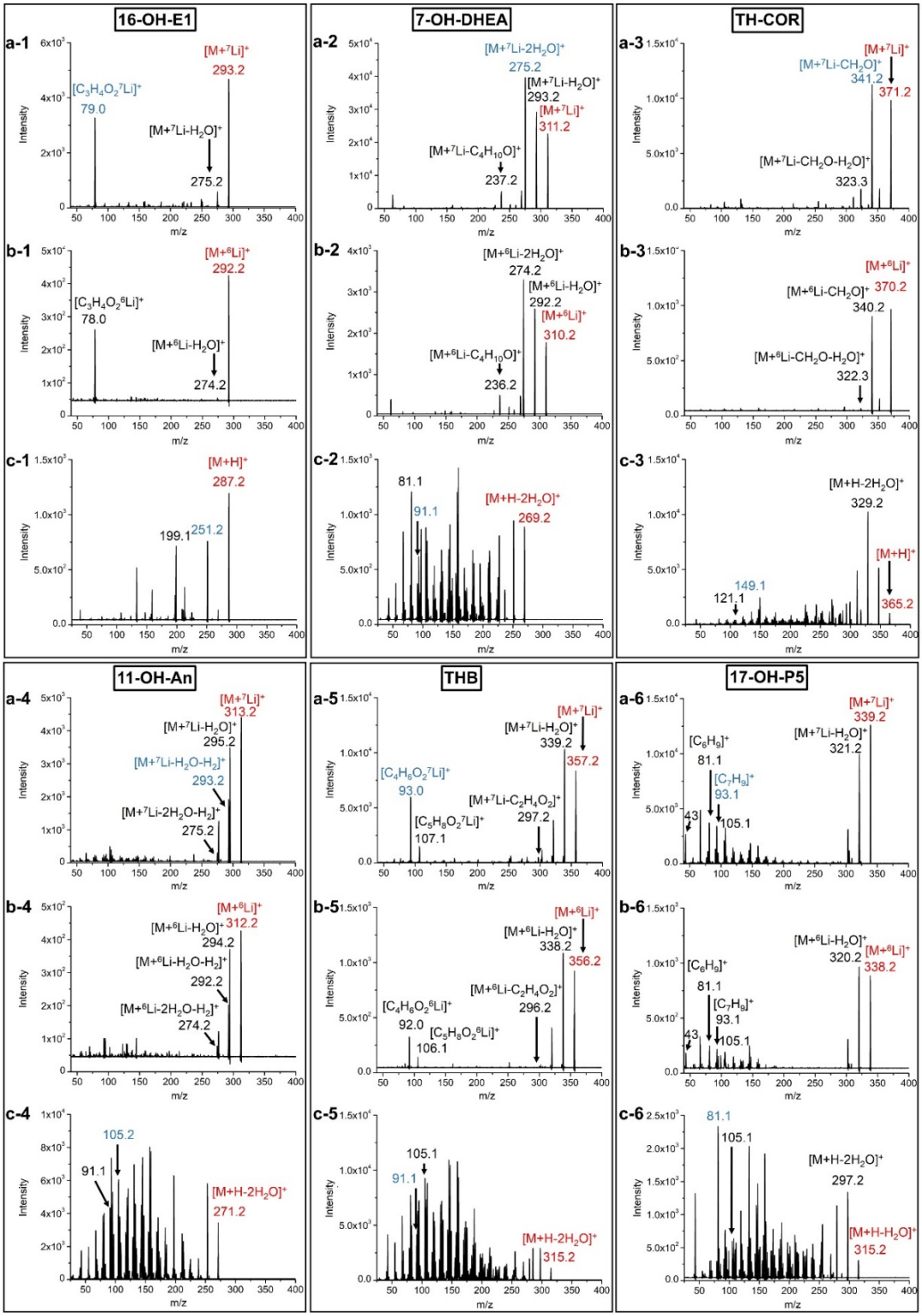


Figure I.8. Product ion spectra of the 3-OH steroids. The precursor ions are $[M+^{7}Li]^{+}$ (a) and $[M+^{6}Li]^{+}$ (b). The mobile phase consisted of: solvent A: 0.2 mM LiCl in deionized water (a and b) and 0.1% FA in deionized water (c) and solvent B (MeOH). 1:16-OH-E1, 2: 7-OH-DHEA, 3: TH-COR, 4: 11-OH-An, 5: THB, 6: 17-OH-P5. The amount of steroid injected was: 16-OH-E1

and 7-OH-DHEA, 1 ng TH-COR and THB, 1 ng for a and b and 20 ng for c 11-OH-An and 17-OH-P5, 20 ng. The analytes were introduced through a two-way valve directly to the MS in 20% of solvent B. The m/z of the product ions in each spectra reflected the ion forms assigned to the structures. The red ions represent the precursor ions for the product ion scan. The blue ions represent the product ions used for the quantitative analysis by MRM.

Prominent fragment ion peaks were observed for 16-OH-E1 at m/z 79.0 (Fig. I.8), which were determined to be lithiated by comparison with the MS/MS from the ^{6}Li-adduct (Fig. I.8a-1 and I.9b-1), and were produced by the cleavage of the D ring (Fig. I.9a). In the case of 7-OH-DHEA and 7-OH-P5, the loss of (H_2O+C_4H_8) from the A ring was found (Figure I.9b and Table I.3), which were confirmed by MS/MS of the ^{6}Li adduct (Fig. I.8a-2 and I.8b-2). $[M+Li-CH_2O]^+$ was the most intense peak for TH-COL, TH-COR, and THS. These steroids contain an OH group at the 17 position, which promotes the loss of CH_2O at position 20 (Fig. I.9c). Dehydrogenation was observed only for 11-OH-An following dehydration, and gave a $[M+Li-H_2O-H_2]^+$ ion (Fig. I.9d). THB, APD and TH-DOC showed prominent peaks at m/z 93 and m/z 107, which could be assigned, respectively, to $[C_4H_6O_2Li]^+$ and $[C_5H_8O_2Li]^+$ and were derived from the D ring (Fig. I.9e). The m/z 297.2 peak was produced by the loss of $C_2H_4O_2$ at the 17 position. P5 and 17-OH-P5 gave fragment ions at m/z 93.0 (Fig. I.9f), but this fragment was obviously different from that of corticosteroids since it was not shifted in the MS/MS from the ^{6}Li-adduct precursor, suggesting that this fragment did not contain lithium and originated from the cleavage of the A and B rings (Fig. I.8a-6 and I.8b-6).

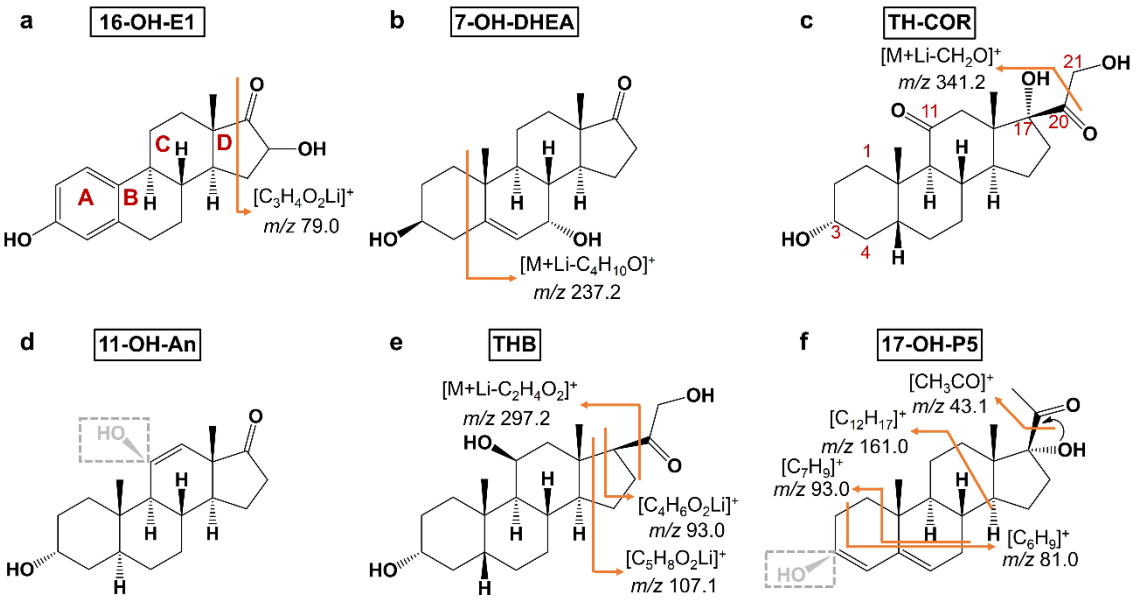
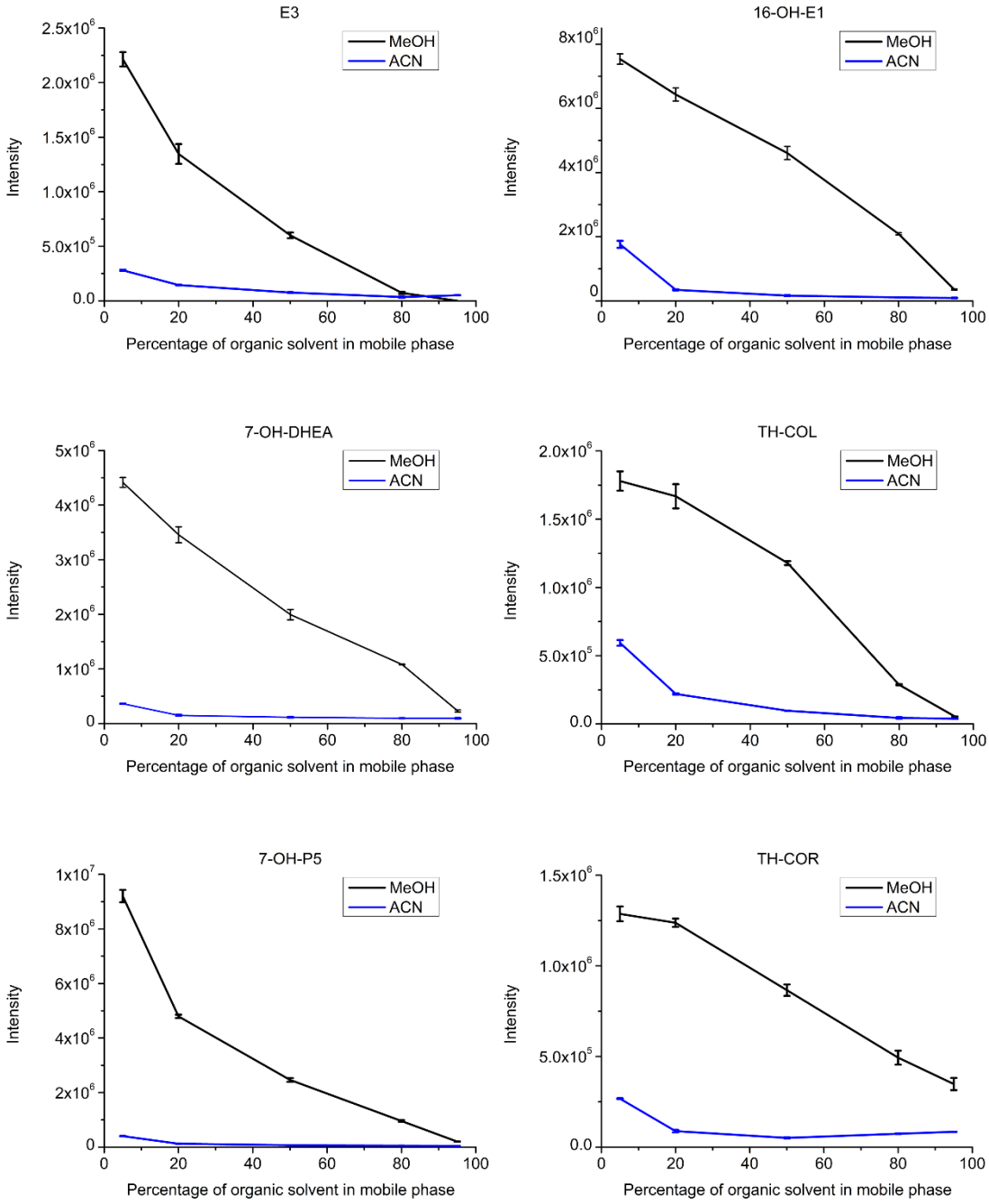


Figure I.9. Fragmentation behavior of lithiated 16-OH-E1 (a), 7-OH-DHEA (b), TH-COR (c), 11-OH-An (d), THB (e) and 17-OH-P5 (f).

I.3.3 Chromatography

MeOH was used as the mobile phase for separating the lithiated 3-OH steroids and gave good separation and higher intensity for 3-OH steroids, over acetonitrile (Fig. I.10).


E3
MeOH
ACN
Intensity
Percentage of organic solvent in mobile phase
16-OH-E1
MeOH
ACN
Intensity
Percentage of organic solvent in mobile phase
7-OH-DHEA
MeOH
ACN
Intensity
Percentage of organic solvent in mobile phase
TH-COL
MeOH
ACN
Intensity
Percentage of organic solvent in mobile phase
7-OH-P5
MeOH
ACN
Intensity
Percentage of organic solvent in mobile phase
TH-COR
MeOH
ACN
Intensity
Percentage of organic solvent in mobile phase

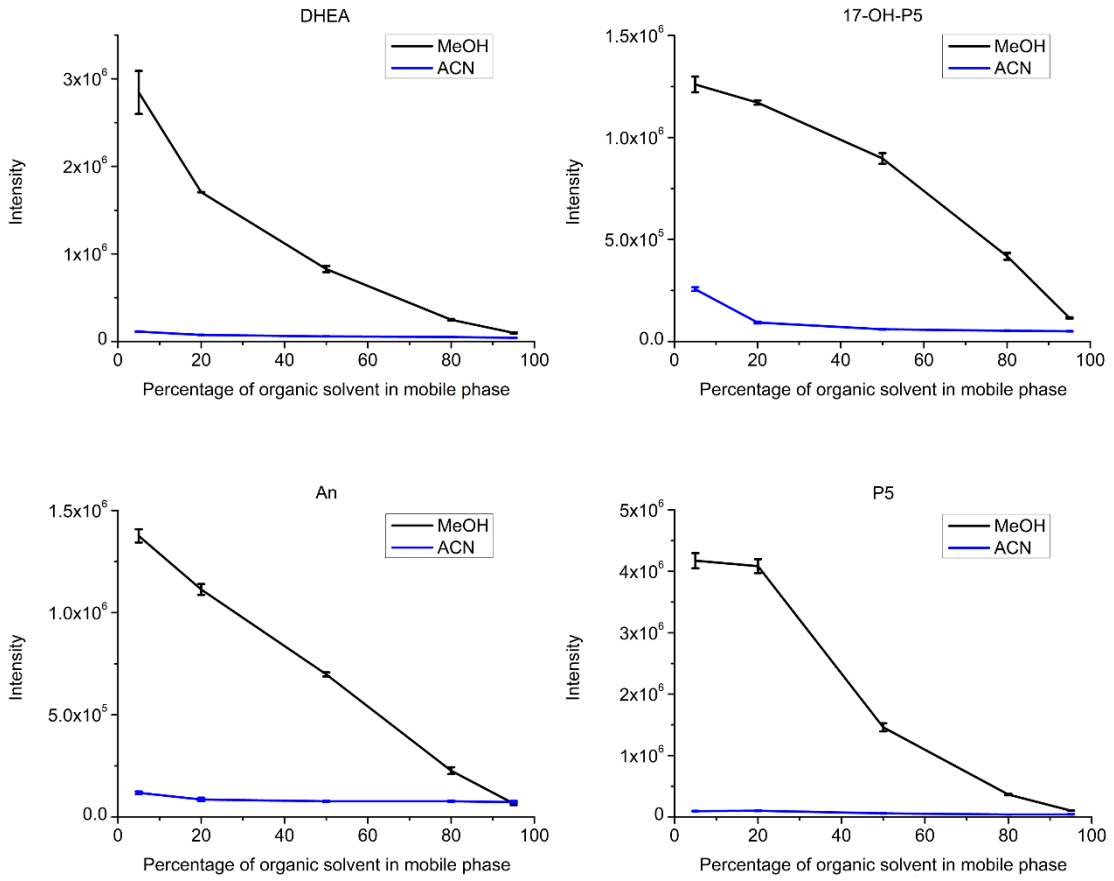


Figure I.10. Effect of the organic solvent used in the mobile phase on the sensitivity of detection for each steroid. The mobile phase consisted of: solvent A (0.2 mM LiCl in deionized water) and solvent B (MeOH or ACN). The steroids (1 ng per injection) were introduced directly to the MS through a two-way valve and detected by the scan mode.

This is partly due to the splitting of the precursor ion into the unique adduct, $[M+ACN+Li]^+$, during the ionization (Fig. I.11). Such an adduct ion was not observed at all in cases where MeOH was used as the solvent, and the degree of formation of adduct ions varied, depending on the specific steroid.

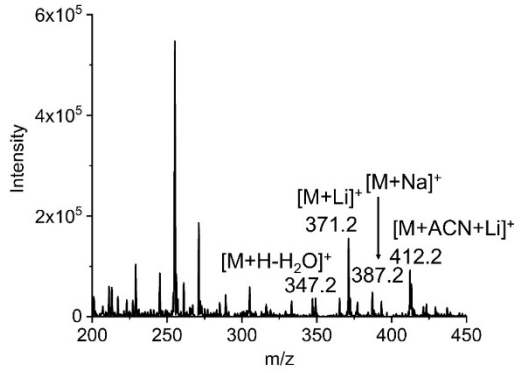


Figure I.11. ESI-MS spectrum of TH-COR obtained by ACN as the organic mobile phase. The 200 ng of TH-COR on column was infused into the MS with 0.2 mM LiCl in 20% aqueous ACN.

When 0.1 mM LiCl in H_2O as mobile phase A and MeOH as mobile phase B were used, the retention time for each steroid was retarded, indicating that the acidic solvent system results in species that are relatively more hydrophilic than those produced in the neutral solvent system (Fig. I.12).

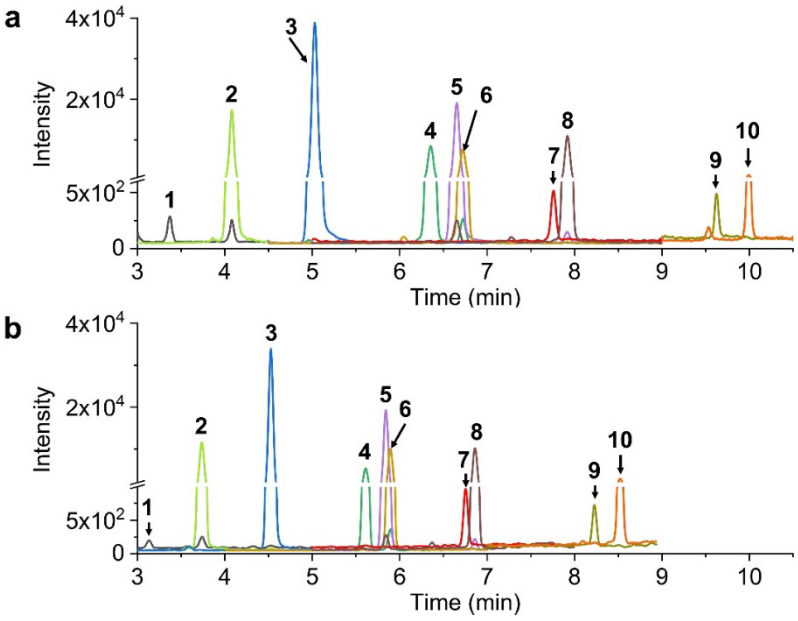


Figure I.12. Chromatograms of ten different steroids at 200 pg on column. The mobile phase consisted of: (a) solvent A (0.1 mM LiCl in deionized water) and solvent B (MeOH) (b) solvent A (0.1% FA in deionized water) and solvent B (MeOH), with 0.1 mM LiCl as auxiliary liquid. The flow rates of the main pump and the auxiliary pump were 0.4 mL min^{-1}. The presence of formic acid in the mobile phase in (b) promoted the earlier elution of the steroids, resulting in a higher H_2O% in the eluate. In addition, the introduction of additional water in the eluate by the

auxiliary pump also increased the H_2O% in the system. When the H_2O% in the spray increased, the intensity of the steroids increased (see Fig. I.10). 1. E3, 2. 16-OH-E1, 3.7-OH-DHEA, 4.TH-COL, 5. 7-OH-P5, 6. TH-COR, 79. DHEA, 8. 17-OH-P5, 9. An, 10. P5.

It is noteworthy that the use of a lower percentage of organic solvent resulted in an improved ionization efficiency for all of the steroids examined in this study (Fig. I.10). Thus, in order to prevent the steroids from being eluted with higher concentrations of mobile phase B, the aqueous lithium ion-containing solution was mixed into the effluent after the column via an auxiliary pump. In view of the increased sensitivity of the steroids, post-column mixing of an equivalent volume of aqueous solution to that of the separation pump decreased the percentage of organic solvent by half (see I.2.3 LC-MS/MS), and thereby increased overall detection sensitivity (Fig. I.10). The mixing of the solvents from the main and auxiliary pumps could be efficiently achieved by using two in-line filters (Agilent 1290 inline filter, 0.3µm), connected in tandem, after the T-piece which connects the two flows. In addition, several concentrations of lithium, ranging from 0.05 to 1 mM of LiCl, were examined in order to produce lithiated forms more efficiently by using the same flow rate as that of the main pump. As a result, nearly all of the lithium adduct ions of steroids could be observed as the predominant species with no less than 0.2 mM LiCl (Fig. I.13 and Fig. I.7). This suggests that the 0.1 mM LiCl in the spraying droplets is sufficient to allow all of the 3-OH steroids to stably form lithium adducts during ionization.

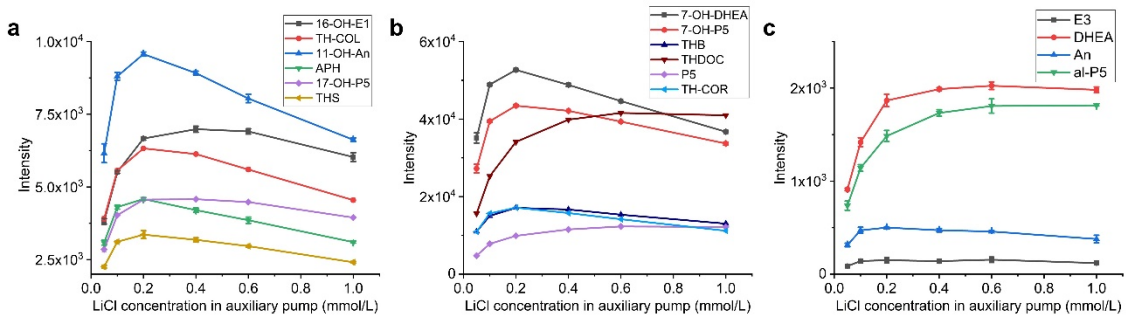


Figure I.13. Effect of different concentrations of LiCl from the auxiliary pump. The mobile phase consisted of: (a) solvent A (0.1% FA in deionized water) and solvent B (0.1% FA in MeOH), with LiCl solution as the auxiliary liquid. The steroid concentrations were at 500 pg on column and were detected by the MRM mode.

Note that Li ions could also be supplied with LiOAc, however, soon after mixing with the FA (0.1%) solvent, the counter anion of AcO^- was readily replaced with $HCOO^-$ to form LiOOCH, which had a propensity to be associated with steroids to form $[M+(LiOOCH)+Li]^+$ (Fig. I.14), the

degree of which varied depending on the specific steroid. This adduct ion was not observed at all in cases when LiCl was used, thus allowing 3-OH steroids to be observed with a higher intensity (see Fig. I.15).

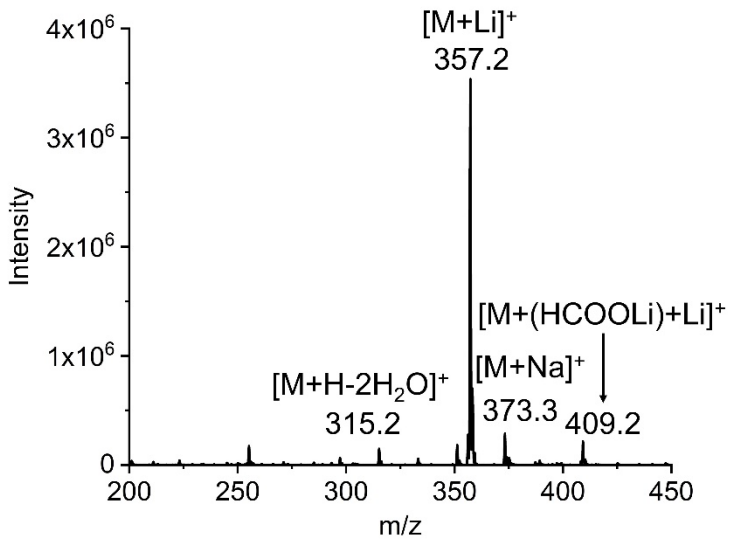


Figure I.14. ESI-MS spectrum of THB obtained by 0.2 mM LiOAc as the auxiliary liquid. The sample (200 ng) was infused into the MS with 0.1% FA in 20% aqueous MeOH and measured by the scan mode.

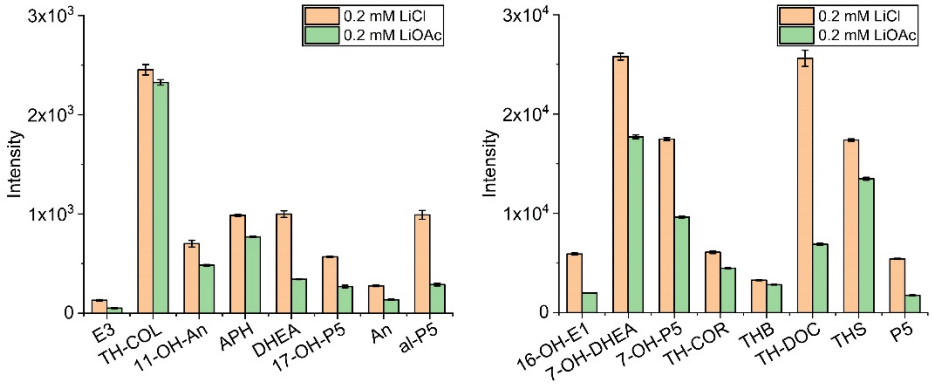


Figure I.15. Comparison of ion intensities of sixteen steroids between adducts with LiCl and LiOAc. The mobile phase for the separation, which consisted of solvent A (0.1% FA in deionized water) and solvent B (0.1% FA in MeOH), was mixed with 0.2 mM LiCl or 0.2 mM LiOAc solution as auxiliary liquid (see EXPERIMENTAL SECTION). The concentration of each steroid was 500 pg on column and were detected by the MRM mode.

I.3.4 Features of high sensitivity

To compare the sensitivity of the developed method (Li method) with the conventional method (H method), standard working solutions were used to check the linearity, LOD (limit of detection), LOQ (limit of quantitation) and detection sensitivity enhancements. LOD and LOQ were obtained at a signal to noise ratio (S/N) of 3 and 10, respectively. As shown in Table I.5, the linearity was satisfactory and the r^2 (linear correlation coefficient) was in excess of 0.9938. The detection sensitivity fold was the ratio of the LOD for the "H method" over the LOD for the "Li method". The LOD (signal to noise ratio (S/N) =3) and LOQ (S/N = 10) of the standard solution was determined with (Li method) and without (H method) the use of a LiCl solution. The detection sensitivity enhancement (fold) was defined as the ratio of the LOD obtained by the "H method" over that by the "Li method". The sensitivity for 16-OH-E1, 7-OH-DHEA, TH-COL, 7-OH-P5, TH-COR, 11-OH-An, APD, THB, 17-OH-P5, TH-DOC, THS, DHEA and P5 were enhanced by 1.53-188 times by the "Li method" (Table I.5).

Table I.5. Calibration curves, linearity range, LOQs, LODs, and sensitivity enhancements for steroids in standard solution obtained with the auxiliary pump on (Li) and off (H).

No	Analyte	Linearity range/pg	Regression curve			LOQ (S/N = 10) /pg		LOD (S/N = 3) /pg		Detection sensitivity enhancement (fold)
			k	b	r^2	Li	H	Li	H	
1	E3	500-5000	0.02	0.05	0.9956	241.5	17.9	72.5	5.4	0.07
2	16-OH-E1	5-5000	1.01	0.19	0.9992	2.2	7.4	0.7	2.2	3.14
3	7-OH-DHEA	1-1000	8.12	0.47	0.9991	0.4	3.6	0.1	1.1	8.33
4	TH-COL	5-5000	0.35	0.04	0.9991	1.6	313.3	0.5	94.0	188.00
5	7-OH-P5	2-3000	2.25	0.16	0.9992	1.2	15.0	0.3	4.5	15.00
6	TH-COR	2-2000	0.86	0.02	0.9990	1.6	49.3	0.5	14.8	29.56
7	11-OH-An	5-5000	0.07	0.01	0.9991	3.2	22.2	1.0	6.5	6.82
8	THB	10-5000	0.42	0.03	0.9996	3.9	34.9	1.2	10.5	8.91
9	APD	10-5000	0.24	0.02	0.9994	5.8	11.7	1.7	3.5	2.02
10	DHEA	50-5000	0.70	-0.02	0.9975	42.2	113.7	12.7	34.1	2.69
11	17-OH-P5	100-2000	0.18	-0.04	0.9965	92.1	164.4	27.6	49.3	1.78
12	TH-DOC	2-2000	1.93	0.01	0.9997	0.7	13.6	0.2	4.1	20.50
13	THS	5-5000	2.09	-0.16	0.9992	0.5	28.5	0.2	8.6	54.08
14	An	200-5000	0.06	0.06	0.9938	111.6	13.0	33.5	3.9	0.12

15	P5	100-5000	0.38	0.10	0.9941	33.9	52.0	10.2	15.6	1.53
16	al-P5	100-5000	0.23	-0.02	0.9945	75.0	8.5	22.5	2.6	0.12

These steroids contain at least one carbonyl and two hydroxyl groups or one carbonyl, one hydroxyl groups and olefinic double bond. The sensitivities for E3, An, and al-P5 were decreased by about one tenth, which can be attributed to the poor formation of fragment ions. The structure of 16-OH-E1 is similar to that of E3, but its sensitivity was increased by 3.14 times. This can be attributed to the fact that 16-OH-E1 contains a carbonyl group at the 17 position, which has a relatively higher affinity for Li^+ than a hydroxyl group and therefore produced an intense fragment ion at m/z 79.0 (see Fig. I.4). In addition, compared with 11-OH-An, An itself contains only a single hydroxyl group, thus resulting in a lower abundance of the dehydrated ion, $[M+Li-H_2O]^+$ upon collision-induced dissociation. The al-P5 molecule, which lacks an olefinic double bond at the 5 position, gave a lower intensity of $[M+Li-H_2O]^+$, compared with P5, since al-P5 is unable to form conjugated double bonds after dehydration. The results for P5 and DHEA, both of which contain a single hydroxyl group but could form conjugated double bonds upon dehydration, showed slight enhancement by 1.53 and 2.69 fold over those with "H method", respectively. Based on these findings, we conclude that 3-OH steroids that contain at least one carbonyl group and two hydroxyl groups or one carbonyl group and 5-olefinic double bond (13 steroids) would give better results when the "Li method" is used (Table I.5). The thirteen steroids could be detected with a lower background by the "Li method" within nine minutes (Fig. I.16).

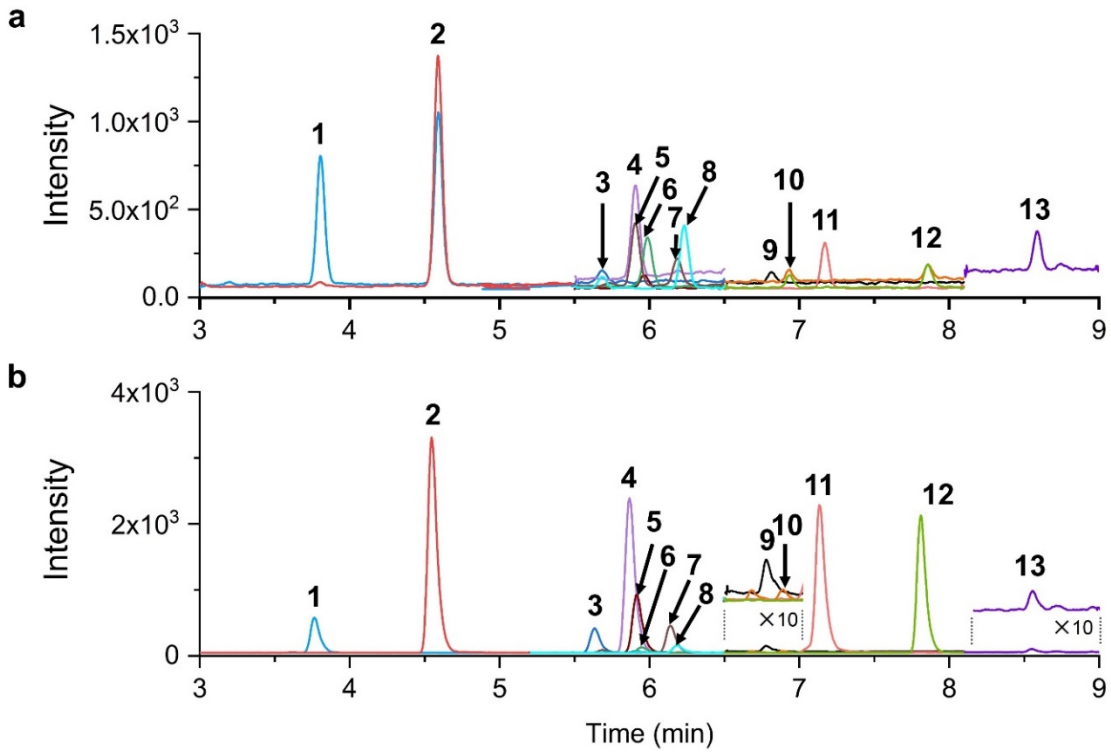


Figure I.16. Chromatograms for the separation of steroids with 50 pg being injected: (a) without LiCl ("H method") (b) with LiCl ("Li method") (see EXPERIMENTAL SECTION). 1: 16-OH-E1, 2: 7-OH-DHEA, 3: TH-COL, 4: 7-OH-P5, 5: TH-COR, 6: 11-OH-An, 7: THB, 8: APD, 9: DHEA, 10: 17-OH-P5, 11: TH-DOC, 12: THS, 13: P5. The MRM transitions used in this analysis were shown in Table I.3 and I.4. The peak traces depicted in colors correspond to those obtained by the transitions indicated in bold, which were used for the quantification.

I.4 Conclusion

In conclusion, the objective of this study was to develop a method for the sensitive and universal quantitation of multi-class 3-OH steroids. By the post-column addition of Li ions into the mobile phase, the sensitivity and selectivity could be remarkably enhanced by 1.53-188 times for 13 different types of 3-OH steroids. As far as the authors' knowledge, this represents the first published method describing the analysis of lithiated steroids by LC-MS/MS. It is noteworthy that the "H method" had a higher sensitivity for An, E3, and 3-keto steroids. In addition, since the analytical settings for the "H method" and the "Li method" are quite similar, they could be

simultaneously operated in the same system, which would allow for a more comprehensive analysis of multi-class steroids in biosamples.

II. Sample pretreatment procedure for isolating the steroids from a mouse brain tissue and profiling of 3-OH steroids using Li ion adduction method (Li-method)

II.1. Introduction

Detection of steroids in brain tissue meets a number of technical difficulties including the high lipid content, the high spatial heterogeneity low steroid concentrations in some cases, and rapid changes in steroid production and metabolism. In this case, the methodological optimization is required to overcome these technical difficulties.

The sample pretreatment procedure from the mouse brain tissue comprises two steps, extraction and purification. The tissue was homogenized to release and dissolve the target compounds into the extraction solvent and the supernatant was collected. Purification aiming to eliminate the contaminants like lipids from the extract is often achieved by solid phase extraction. [30, 44, 56] The flowchart of the strategy is described in Fig. II-1. The samples were weighed in a centrifuge tubes. Then, internal standards, which will be required for normalization of each analyte, were added to the tube. Then, extraction solvent was added and the samples were homogenized. The mixture was centrifuged, the supernatant was collected and evaporated to dryness. The resultant residues were re-dissolved in the solvent used for the following step, and loaded on a pre-treated solid-phase-extraction (SPE) cartridge, which is normally comprised of reversed phase materials like C18 silica gel, by which most of the steroids could be eluted with some organic solvent containing solution according to their hydrophobicity [28, 57]. The sample was then washed with the washing solution and collected with the eluting solution. Finally, the sample was evaporated, dissolved, and introduced into the instrument for analysis.

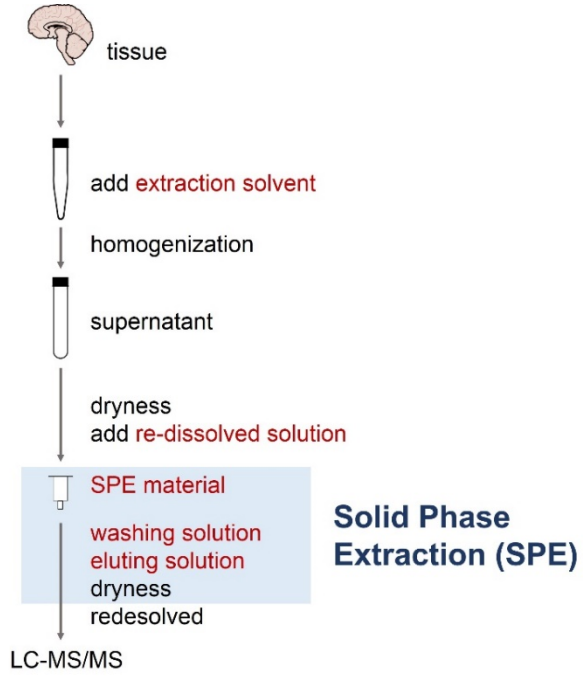


Figure II.1. Flow chart for the sample preparation from mouse brain tissue.

Since the steroids are quite hydrophobic, the extraction solutions are mainly low polar organic solvents including methanol, ethanol, acetone and ethyl acetate (EtOAc) or the mixture of these solvents. Formic acid (FA) or acetic acid (AcOH) was also contained to adjust the pH. The extraction solutions were listed in Table II.1. Due to the high hydrophobicity of the steroids, the re-dissolved solution should not be very hydrophilic. Since the amount of steroids in brain tissue is relatively low, compared with the level of other lipids, some of which contain amino or carboxyl groups and could be efficiently removed using ion-exchange resin packed cartridge.[44, 56] After the sample is loaded to a SPE cartridge, it should be exhaustively washed to remove interfering substances while steroids are maintained. The choice of washing and eluting solution, taking into consideration the tissue type, contaminants and the SPE type, is the key for the high recovery of target analytes. After washing, steroids are slowly eluted off the sorbent. The sample preparation condition for mouse brain is summarized in Table II.1.

Table II.1. Sample preparation condition for mouse brain.

analyte	tissue	extraction solution	re-dissolved solution	SPE material	washing solution	eluting solution	References

AE, DHAn, DHEA, DHT, E1, βE2, P4, P5, Test,	mouse adrenal gland, testicle, dorsolateral prostate gland and ovary	MeOH	5% MeOH	Oasis HLB 3cc	5% MeOH	MeOH	[30]
DHEA , DHP4	rat brain Cerebral cortex and hippocampus	0.1% AcOH, MeOH		Hybrid-SPE Cartridges	0.1% AcOH, MeOH	0.1% AcOH, MeOH	[56]
Cort, COL, DHEA, DHP4, E3, THDOC, isoP4, P4, P5, Test	mouse brain hippocampus and hypothalamus	ethanol/ acetone (1:1, v/v)	300 μL of ethanol/ acetone (1:1, v/v)/H_2O mixture (7:3 v/v), diluted with 10 mL of $_2O$	cation exchange cartridge	0.1 M HCl (1 mL), H_2O	ACN/ MeOH (1:1 v/v,)	[44]
17-OH-P4, 17-OH-P5, AE, AL, COL, COR, Cort, DHAn, DHEA, DHT, DOC,DOS, E1, αE2, βE2, P4, P5, Test,	Gonad tissue	75% MeOH, H_2O	2 mL 75% MeOH, H_2O, 9mL H_2O	Bond elute C18	25% MeOH, H_2O	80% MeOH, H_2O	[57]

11-keto-Test							
P5, P4, DHP, THP4, Test, DHT, DHAn, αE2 and β-E2	cerebral cortex, cerebellum, spinal cord and brachial nerve	0.1% AcOH, MeOH	10% MeOH, H_2O	Discovery DS-C18	10% MeOH, H_2O	MeOH	[28]
COL, Cort	rat and mouse brain	EA	5% MeOH, H_2O	C18	H_2O	MeOH	[58]
7α-OH-DHEA, 7β-OH-DHEA, DHEA	rats brain	50 mM citrate phosphate buffer (pH 8.6) and 3 mL EtOAc for LLE, EtOAc layer was kept	pertoleum ether and 80% MeOH, H_2O for LLE. MeOH, H_2O layer was kept				[59]
CORT, THB	rats brain	0.1% AcOH, MeOH	dilute with 3 times of H_2O	Strata-X cartridge	H_2O, 50% MeOH, water and hexane–EA (5:1,v/v)	ethyl acetate–hexane (7:3, v/v)	[10]

Liquid–liquid extraction (LLE) with organic solvents has also been used to isolate steroids from tissues. However, LLE can also extract other hydrophobic substances, such as lipids. In this case, LLE is often coupled with SPE to extract the steroids from tissue.

The coupled use of SPE is also a strategy to get rid of the contaminants in tissue, where different SPE material could adsorb different compounds. [60, 61] William J. Griffiths et. al developed a strategy by using the reversed phase SPE, cation exchange SPE and anion exchange SPE [60].The extract first passed through a C18 SPE, followed by a cation exchange and anion exchange SPE. The eluted steroids were derivatized to form oximes, and then, the derivatives were separated with a C18 column followed by another cation exchange column. However, this sample pretreatment procedure, containing five SPE and one chemical derivatization steps, is time-consuming and causes sample loss.

In this chapter, I optimized the sample pretreatment procedure for isolating the steroids from a mouse brain tissue and analyzed them using Li ion adduction method established in Chapter I.

II.2 Material and Methods

II.2.1 Chemicals

Analytical-reagent-grade acetate acid (AcOH), ethyl acetate (EtOAc) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (Tokyo, Japan). The HF Bond Elute C18 (1 mL, 60 mg) were obtained from (Agilent Technologies, Inc. Santa Clara, CA). The other chemicals used in this chapter were described in Chapter I.

II.2.2 Preparation of standard stock, calibration, and quality control solutions

The preparation of working standard solutions was described in Chapter I, which were prepared at concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, and 500 ng/μL with 40% MeOH/H_2O. For calibration and quality control samples, 24 μL of blank matrix (see below) was spiked with 12 μL of working standard solution and 4.8 μL of IS solution. The samples were evaporated to dryness and re-dissolved in 12 μL of 40% MeOH/H_2O. Finally, the calibration samples were at levels of 0.04, 0.08, 0.2, 0.4, 0.8, 2, 4, 8, 20, 40, 80, 200 pg/mg.

II.2.3 Animals

All animal experiments were performed in compliance with the Animal Experimental Committee of the Institute for Protein Research at Osaka University. C57BL/6NJcl mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). Mice were maintained in a quiet environment with the temperature controlled at 24 ± 1°C. Mice were on a 12h light/dark cycle with free access to standard chow and water at all times. Mice, at the age of 7 weeks, were deeply anesthetized with isoflurane, and blood samples were obtained by via cardiac puncture. The mice were subsequently sacrificed by decapitation and whole brains were quickly removed from the scull. The removed whole brains were immediately frozen in liquid nitrogen and stored in a -80°C freezer until further used.

II.2.4 Sample preparation

Samples thawed on ice. A 7.5 mL aliquot of 0.25 M AcOH was added per gram of sample. Brains were homogenized in a Nippi (Tokyo, Japan) Biomasher SP disposable pestle with a Power masher electronic attachment. After thawing, the brain tissues were placed in the pestle and homogenized while buffer was added incrementally. After the entire amount of buffer was added and the mixture homogenized thoroughly, the homogenate was sonicated with a TAITEC (Tokyo, Japan) VP-050 (Settings PWM 30%, on: off cycle 4:1 sec) for 120 sec. Samples were frozen until further use.

The scheme for the extraction of steroids from brain tissue is shown in Fig. II.2. After homogenization, the homogenate was divided into three parallel samples and transferred to 10 mL-glass tubes for liquid-liquid extraction. Internal standards (6 µL of a mixture of 200 pg/µL TH-COR-d_6 and P5-$^{13}C_2$, d_2 and 400 pg/µL of 16-keto-E2-d_5, and DHEA-d_5 in 40% MeOH) were added to each brain sample. A 3000 µL of EtOAc were added into the tube, and solid NaCl was added until saturation. The mixture was then vortexed for 10 min at 1800 rpm and centrifuged for 10 min at 3000 rpm. The organic layer was collected and the remaining matrix was re-extracted two times using the above procedure. The three combined organic layers were then dried under a nitrogen stream at room temperature. The subsequent residue was re-dissolved in 400 µL of 50% ACN/H_2O and diluted with 1600 µL of ultrapure water. The supernatant was loaded on a Bond Elute, which was pretreated with 2 mL 80% ACN/H_2O and 2 mL 10% ACN/H_2O, followed by washing three times with 1000 µL of 10% ACN/H_2O and eluted with 1000 µL of 80% ACN/H_2O. The eluate was evaporated to dryness with a speed-vac and the resulting solid re-dissolved in 12 µL of 40% MeOH/H_2O.

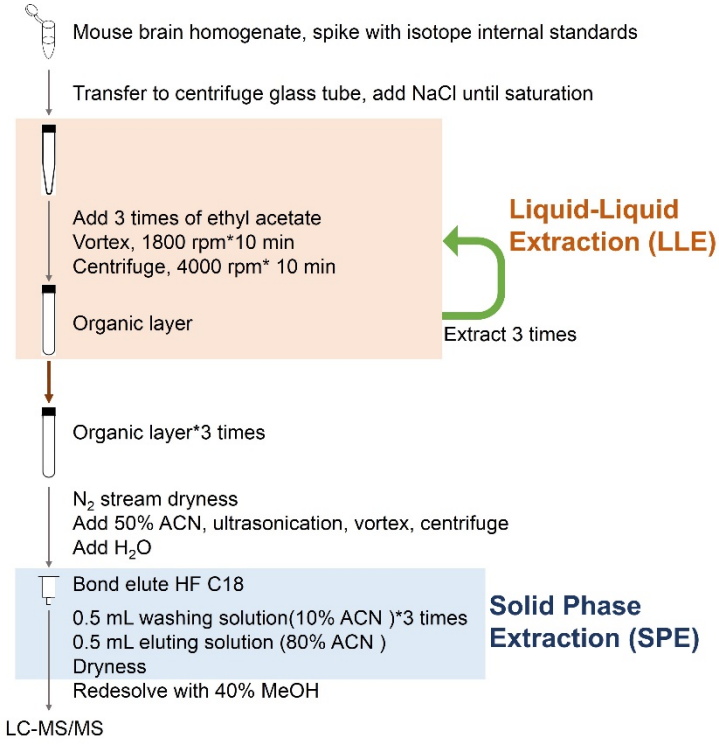


Figure II.2. Scheme for the extraction of steroids from brain tissue in this analysis.

II.2.5 Blank matrix

For method validation, charcoal was added during the sample preparation procedure. Mouse brain samples were homogenized and extracted three times with 3 mL of EtOAc (v/v). After evaporating the EtOAc under a nitrogen stream and re-dissolving, charcoal was added to strip the steroids from the system. 0.8 g of activated charcoal was added per gram of tissue, followed by vortexing for 10 min and centrifugation for 10 min. The supernatant was loaded on the bond elute column for further purification. The eluate was evaporated to dryness with speed-vac and re-dissolved with 40% MeOH/H_2O to form 1.25 mg μL^{-1} of blank matrix.

II.2.6 Instruments

The Cute Mixer CM-1000 was purchased from EYELA (Tokyo, Japan) and the Portable Centrifuge NT-8 was obtained from NI-TION (Tokyo, Chiba). The LC-MS/MS system and MRM parameters were as described in Chapter I.

II.3 Results and Discussion

II.3.1 Optimization of sample pretreatment procedure

To optimize the liquid-liquid extraction (LLE) conditions, the standard solution (200 pg μL^{-1} in 20% MeOH, 10 µL) was transferred to a 10 mL glass centrifuge tube and 190 µL H_2O was added and the pH was adjusted to 3 with AcOH. Analytes were extracted by adding 1000 µL of diethylether, EtOAc or ACN to the tube. NaCl was added to form a two-layer system. As shown in Fig. II.3, EtOAc gave the best extraction efficiency with 96.5-101.5% and the lowest CV%. The recovery from acetonitrile was also satisfactory, but there were numerous NaCl particles on the wall after the organic layer had been dried.

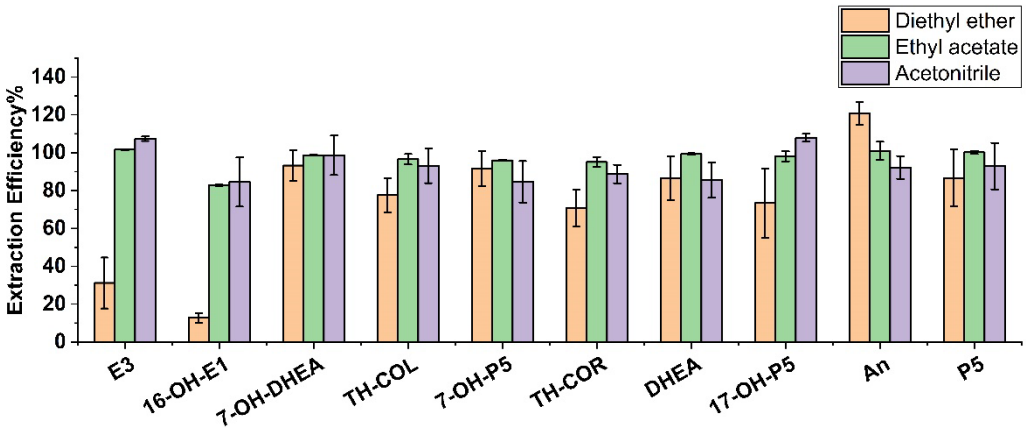


Figure II.3. Comparison of different organic solvents in LLE. The concentration of the steroids were 500 pg in each analysis and detected by MRM mode. The extraction efficiencies were calculated with the peak area of the same amounts of standards without extraction as the base values.

Next, in order to optimize the ratio of EtOAc over H_2O, the same amount of standard solution as the above was used. Three times of EtOAc extractions gave a good recovery, as shown in Fig. II.4. The standard solution (200 pg for each, prepared with the same washing solution) was transferred to the solid phase extraction (SPE) C18 column which was activated with 2 ml of eluting solution and washed with 2 ml of washing solution prior to use. The material in the activated column was rinsed with 3 ml of washing solution to remove impurities. Finally, the column was eluted with 1 mL of eluting solution.

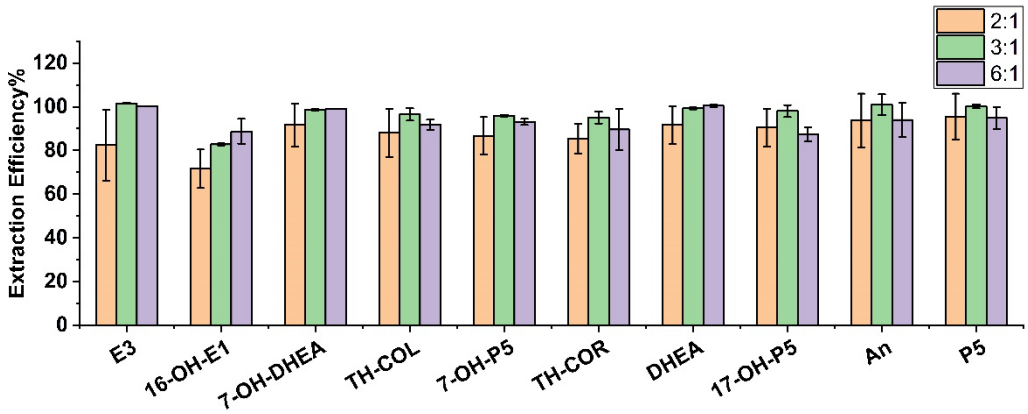


Figure II.4. Comparison of different ratios of EtOAc over H_2O in LLE. The concentration of the steroids were 500 pg in each analysis and detected by MRM mode. The extraction efficiencies were calculated with the peak area of the same amounts of standards without extraction as the base values.

Next, I optimized washing and eluting solutions. The data shown in Fig. II.5a and 5b indicated 10% and 80% ACN/H_2O, respectively, to give the most extraction efficiencies, which were calculated with the peak area of the same amounts of standards without extraction as the base values.

Three volumes of ethyl acetate were used in LLE while 10%, 10% and 80% ACN was used as the washing, loading and eluting solution in SPE. Overall yields of the ten kinds of steroids, obtained with the above extraction procedure, were 88-107%. The recovery of the spiked steroids from the mouse brain sample was examined in "II.3.2 Method validation".

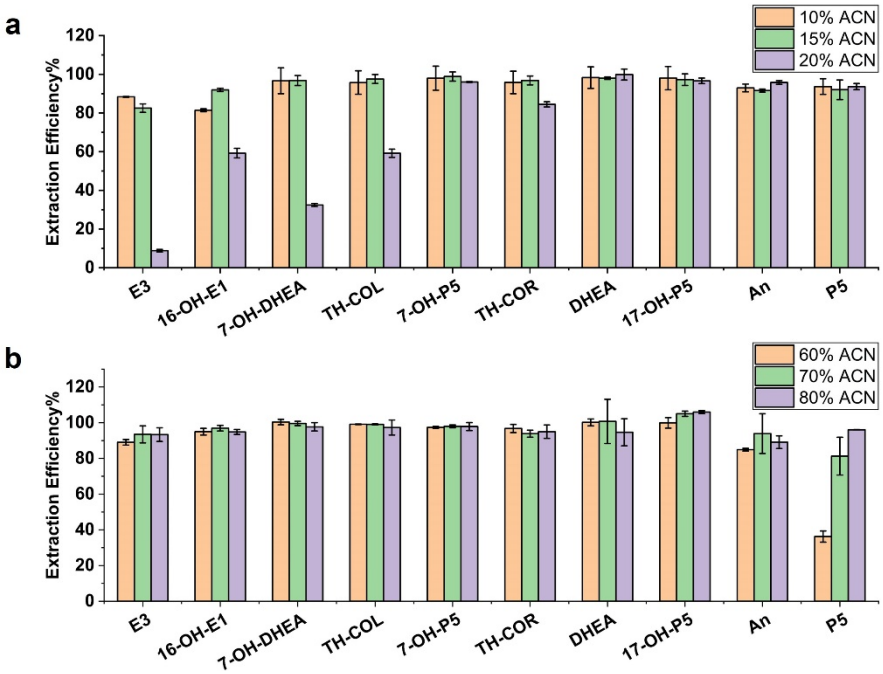


Figure II.5. Sample pretreatment optimization. The concentration of the steroids were 500 pg in each analysis and detected by MRM mode. (a) Comparison of different washing and loading solutions in SPE. (b) Comparison of different eluting solution in SPE. The extraction efficiencies were calculated with the peak area of the same amounts of standards without extraction as the base values.

II.3.2 Method validation

The developed method was satisfactorily validated in terms of the limit of quantification (LOQ), linearity range, extraction recovery, precision, and accuracy.

Calibration curves and LOQ

The calibration curves, correlation coefficients, linearity ranges, and LOQs of the 13 steroids in the spiked blank matrix are shown in Table II.2. The calibration curve was constructed using the peak area ratios of a compound to IS versus the ratios of concentrations of a compound at different levels to the concentration of IS. The correlation coefficient square (r^2) was calculated. LOQ was tested at a signal to noise (S/N) of 10. Good linearity was observed for all 13 steroids within the ranges (0.08-80 pg mg^{-1} for 16-OH-E1, 7-OH-DHEA, 7-OH-P5, TH-COR, and 11-OH-An 0.2-80 pg mg^{-1} for TH-DOC 0.2-200 pg mg^{-1} for TH-COL, THB and THS 0.8-200 pg mg^{-1} for APD 2-200 pg mg^{-1} for DHEA, 17-OH-P5 and P5). The linear correlation coefficient square (r^2) was greater than 0.9945. The lower LOQ (LLOQ) was 0.024-1.964 pg mg^{-1} in mouse

brain, suggesting that the developed method is highly sensitive for the quantification of 3-OH steroids.

Table II.2. Calibration curves, linearity range, and LOQs of steroids in a blank matrix obtained for the developed method.

No.	Analyte	LOQ (pg mg^{-1})	Slope	Intercept	r^2	Linearity range (pg mg^{-1})
1	16-OH-E1	0.058	1.795	0.073	0.9996	0.08-80
2	7-OH-DHEA	0.024	9.479	0.222	0.9993	0.08-80
3	TH-COL	0.113	0.406	0.038	0.9990	0.2-200
4	7-OH-P5	0.045	2.911	0.191	0.9996	0.08-80
5	TH-COR	0.066	0.976	0.011	0.9993	0.08-80
6	11-OH-An	0.030	0.089	0.012	0.9992	0.08-80
7	THB	0.016	0.531	0.020	0.9998	0.2-200
8	APD	0.453	0.27	0.02	0.9995	0.8-200
9	DHEA	1.540	0.650	0.069	0.9959	4-200
10	17-OH-P5	1.231	0.169	0.014	0.9951	4-200
11	TH-DOC	0.039	3.603	1.000	0.9991	0.2-80
12	THS	0.024	2.646	0.128	0.9994	0.2-200
13	P5	0.742	0.342	0.238	0.9945	2-200

Matrix effect and recovery

To evaluate the matrix effect (ME) and recovery (R), low (0.2 or 2 pg mg^{-1}), medium (2 or 20 pg mg^{-1}) and high (20 or 80 pg mg^{-1}) concentrations of spiked blank matrix and mouse brain samples (n = 3) were assessed. The matrix effect value was calculated as ME (%) = $A_{matrix}/A_{solution}$ × 100, where $A_{solution}$ is the compound peak area of 10 µL of pure standard and A_{matrix} is the compound peak area of blank matrix spiked with 10 µL standard sample. The recovery value was calculated as R (%) = $A_{pre\text{-}spike}/A_{post\text{-}spike}$ × 100, where $A_{pre\text{-}spike}$ is the compound peak area of mouse brain spiked with 10 µL standards before extraction and $A_{post\text{-}spike}$ is the compound peak area of brain sample spiked with 10 µL standards after extraction. The extraction recoveries (R) and matrix effect (ME) were within the range of 82.00-108.92% and 80.96-116.95%, as shown in Table II.3.

Table II.3. Matrix effect and recovery of 13 steroids in mouse brain.

No.	Analyte	Spiked con.(pg mg^{-1})	Matrix effect%		Recovery%	
			Mean%	RSD%	Mean%	RSD%
		0.2	104.34	8.01	88.34	3.59
1	16-OH-E_1	2	95.95	5.52	84.51	5.97
		20	100.18	2.04	82.70	5.65
		0.2	113.69	8.16	95.20	6.25
2	7-OH-DHEA	2	101.34	1.48	99.70	5.09
		20	105.36	0.60	100.82	4.25
		0.2	111.26	8.21	105.65	12.92
3	TH-COL	2	101.22	2.09	105.03	6.75
		20	106.44	1.01	105.91	2.87
		0.2	100.36	0.95	90.13	5.75
4	7-OH-P5	2	94.45	3.59	82.00	5.13
		20	101.64	3.52	86.27	6.37
		0.2	102.51	4.66	105.25	8.78
5	TH-COR	2	100.90	1.47	99.57	4.13
		20	105.32	0.46	101.06	3.63
		0.2	88.76	1.45	87.24	7.20
6	11-OH-An	2	87.13	5.69	96.78	8.58
		20	100.12	3.45	96.84	4.47
		0.2	80.96	6.68	92.11	11.26
7	THB	2	95.65	3.29	97.81	3.01
		20	102.55	3.01	101.10	4.62
		2	98.94	15.53	106.32	7.65
8	APD	20	107.79	2.71	102.38	5.76
		80	100.73	1.87	94.09	6.37
		8.00	89.83	3.38	102.40	8.15
9	DHEA	20.00	91.23	6.14	90.57	11.01
		80.00	95.14	4.69	89.92	3.02
		8.00	97.58	16.71	93.34	3.90
10	17-OH-P5	20.00	92.99	9.45	89.04	5.74
		80.00	97.12	4.00	92.71	4.67
11	TH-DOC	2	100.06	5.40	101.30	10.59

		20	107.32	2.86	95.18	10.68
		80	98.15	0.75	95.68	7.86
		0.2	82.92	4.12	108.92	10.56
12	THS	2	93.41	0.01	104.99	7.80
		20	106.02	0.02	100.52	10.65
		2	106.78	0.86	95.40	10.68
13	P5	20	105.04	5.48	82.31	5.96
		80	113.82	6.64	95.21	5.96

Accuracy and precision

The accuracy and precision of the method were assessed by performing six replicates of mouse brain samples spiked with low (0.2 or 2 pg mg^{-1}), medium (2 or 20 pg mg^{-1}) and high concentrations (20 or 80 pg mg^{-1}) of steroids (Table II.4). Accuracy was calculated as the averaged percentage for the measured concentrations to the real ones. Precision was expressed as the RSDs (relative standard deviation) of the measured concentrations and was performed on three separate days. The low and high concentrations of steroids spiked in the intra and inter-batch samples were obtained within acceptable ranges. Table II.4 summarizes the accuracy and precision data obtained for mouse brain. The results indicated that the method shows a moderately good accuracy and precision: the accuracy was 79.42-108.0% the intra-day precision was 0.31-13.54% the Inter-day precision was 0.58-13.34%. The method was considered to be suitable for the analysis of the 13 steroids in mouse brain tissue in terms of accuracy and precision.

Table II.4. Accuracy, and intra-day and inter-day precision obtained for the analysis of 13 steroids in mouse brain tissue.

No.	Analyte	Spiked con. (pg mg^{-1})	Mean (pg mg^{-1})	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
		0.2	0.19	96.23	6.73	10.61
1	16-OH-E1	2	1.65	82.72	1.22	5.30
		20	15.88	79.42	6.05	1.36
		0.2	0.19	93.02	5.98	1.60
2	7-OH-DHEA	2	1.81	90.55	5.66	7.85
		20	20.18	100.89	0.31	1.51
	TH-COL	0.2	0.18	89.85	0.99	12.10

3		2	1.83	91.69	2.34	2.63
		20	19.03	95.15	4.71	3.34
		0.2	0.20	99.91	3.71	7.32
4	7-OH-P5	2	1.69	84.30	2.85	1.99
		20	16.40	81.99	8.61	7.08
		0.2	0.18	92.35	1.35	2.25
5	TH-COR	2	1.89	94.54	7.78	1.40
		20	19.27	96.36	6.67	3.96
		0.2	0.20	101.19	9.67	7.28
6	11-OH-An	2	2.05	102.57	6.45	4.61
		20	19.00	95.01	5.28	3.99
		0.2	0.19	94.00	1.46	12.57
7	THB	2	1.97	98.39	4.28	0.58
		20	19.12	95.62	0.74	6.71
		2	2.10	105.00	2.49	9.93
8	APD	20	19.69	98.43	7.15	5.12
		80	82.25	102.81	10.66	3.26
		8	8.00	100.05	1.85	5.40
9	DHEA	20	21.67	108.35	12.44	10.53
		80	74.79	93.49	6.51	10.74
		8	7.57	94.58	9.05	8.80
10	17-OH-P5	20	17.24	86.21	4.72	4.70
		80	68.34	85.43	9.97	0.65
		2	20.17	100.87	2.85	11.12
11	TH-DOC	20	2.08	104.04	13.54	11.30
		80	71.50	89.38	3.07	5.94
		0.2	0.22	108.00	2.72	5.55
12	THS	2	1.81	90.52	11.89	13.34
		20	18.51	92.57	12.74	8.05
		2	1.94	96.86	6.09	6.60
13	P5	20	16.72	83.58	4.42	4.02
		80	66.98	83.73	3.89	8.40

II.3.3 Biosample analysis (mouse brain tissue)

The new method was applied for the analysis of steroids in brain tissue from five healthy male mice. Six steroids were identified and quantified in whole brain samples. The steroids were identified by comparing the retention time and the peak intensity ratio of two MRM transitions of the samples with that for a spiked blank matrix sample. The criteria for the identification was as follows: the relative difference in retention time should be less than 2% the relative difference in the ratio of the peak intensity of two MRM transitions should be less than 20%. The method allowed six steroids to be identified from one mouse brain tissue sample (Fig. II.6). Note that there are shoulders to the THB peak on the right side and to the DHEA peak on the left side(lower panel of Fig. II.6), which were, most probably, endogenous metabolites in the brain extract that were slightly observed for the blank matrix sample (upper panel).

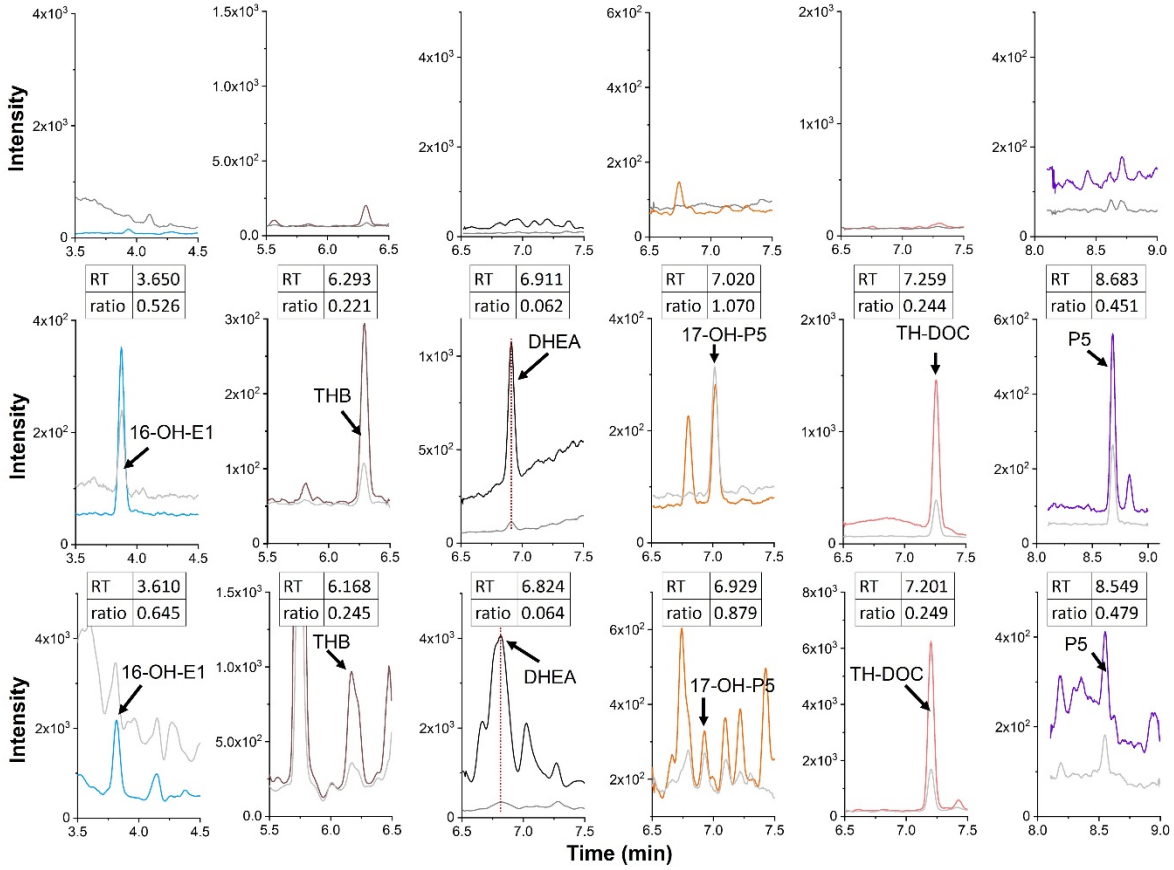


Figure II.6. MRM chromatograms of steroids from a charcoal-treated blank matrix sample (upper), a charcoal-treated spiked sample (medium) and a whole mouse brain (lower). Each steroid was identified using two MRM transitions (Table I.3). The colored traces in each chromatogram are depicted with the values (= transitions) in bold in Table I.3, which were used for the quantification (16-OH-E1: 293.2→79.1, THB: 357.2→93, DHEA: 295.2→277.2, 17-OH-

P5: 339.2→93.1, TH-DOC: 341.2→93 and P5: 323.2→43.1). The gray traces represents the transitions used for the qualification in Table I.3 (16-OH-E1: 293.2→275.2, THB: 357.2→107.1, DHEA: 295.2→105.1, 17-OH-P5: 339.2→81.1, TH-DOC: 341.2→107 and P5: 323.2→105.1). The amounts of steroids spiked to the charcoal treated sample was 50 pg for 16-OH-E1, THB, and TH-DOC, and 500 pg for DHEA, 17-OH-P5, and P5. The ratios indicated in the insets were calculated on the basis of the peak height ratios of the two transitions (normal over bold values, obtained for each steroid, in Table I.3).

As shown in Table II.5, the amount of steroids in the tissue varied from sample to sample, ranging from 0.12 to 27.01 pg mg^{-1}. In earlier studies, DHEA, TH-DOC, and P5 were quantified in whole rat brain at concentrations of 0.05-7 pg mg^{-1} [34, 60, 62, 63]. The level of TH-DOC and P5 in the mouse brain is similar to those in the rat brain. However, in our analysis, the level of DHEA was found to be much higher in the mouse brain tissue. The previous work by Sjövall et al. indicated that the cholesterol autoxidation took place mainly during chemical derivatization for GC-MS, where the high temperature and the existence of catalysts for the derivatization promoted the formation of DHEA and P5 from cholesterol.[64] In our experiment, the sample collection and homogenization was conducted on ice and the extraction was performed at room temperature (see Materials and Methods). Meanwhile, Sirkku et al. found DHEA in the mouse brain, however the concentration was lower than LOQ (2.88 pg/mg) [44]. We might speculate relatively high level of DHEA, obtained in the present study, to be ascribed to the mice species used in the present experiment. THB was detected in the brain of rats that had been subjected to immobilization but not in brains from unstressed rats[10]. This is the first report of the presence of 16-OH-E1, THB, and 17-OH-P5 in an unstressed control mouse brain.

Table II.5. Quantitative results for the steroids of whole mouse brain tissue and a comparison with values reported in previous studies.

Steroid	concentration in whole brain (pg mg^{-1}), n = 5 [a]	Literature concentration in male rat brain (pg mg^{-1})	Literature concentration in male rat cortex (pg mg^{-1})
16-OH-E1	0.97-2.38	-	-
THB	0.117-0.462	1.38 ± 0.54(stressed) [10]	-
DHEA	15.59-27.01	0.05-0.11 [60],	46-79 [56]

		0.2-0.6 [63],	
17-OH-P5	<LOQ	-	-
TH-DOC	0.129-0.535	1 [34]	-
P5	1.64-1.98	0.60-1.2 [60], 7 [62]	12-15 [65]

LOQ: limit of quantitation.

[a]The concentration in each individual brain is shown in Table II.6.

Table II.6. Quantitative results for the steroids detected in each mouse brain tissue (n = 5).

Steroid	Concentration in whole brain (pg mg^{-1}), n = 5				
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
16-OH-E1	1.08±0.18	0.99±0.05	0.97±0.13	1.42±0.29	2.38±0.36
THB	0.117±0.014	0.376±0.023	0.300±0.004	0.462±0.016	0.393±0.034
DHEA	17.31±1.07	18.75±3.48	27.01±5.82	15.59±1.88	17.25±1.36
TH-DOC	0.129±0.002	0.385±0.050	0.237±0.002	0.535±0.037	0.410±0.032
P5	1.68±0.22	1.73±0.20	1.64±0.03	1.98±0.25	1.72±0.23

II.4 Conclusion

It is the first report of the detection of 16-OH-E1, THB, and 17-OH-P5 in a control mouse brain, although the actual role of these steroids in the brain needs to be elucidated and will require further investigation. The method should have a potential for determination of various 3-OH steroids in separate regions of the brain such as the cerebrum, cerebellum, hippocampus, and hypothalamus.

III. Profiling of multi-class steroids in mouse brain by the combination of Proton adduction method (H-method) and Li-method

III.1 Introduction

The 3-OH steroids, which are amenable to the dehydration during MS measurement to make their quantification difficult, have been the analytical target to be solved in the former chapters. Meanwhile, there are another major class of steroids classified as 3-keto steroids, which are synthesized in steroid metabolic pathways as well as 3-OH steroids and could co-exist with 3-OH steroids in biosamples. They have a carbonyl group at 3 position instead of a hydroxyl group (3-OH steroids) and could be stably detected in the protonated form. the combination of proton adduction method (H-method) and Li-method could help us to profile multi-class steroids in a short time.

As shown in Fig.2, corticosteroids, androgens and progestogens has hydroxyl groups while the estrogens shares a 3-phenolic hydroxyl group. In the previous multi-class steroid analysis, the biosamples were often derivatized in sample preparation procedure (see 2.3.2 Chemical derivatization for LC-MS) and then introduced to LC-MS [20, 32]. The introduction of an easily ionized functional group also change the hydrophobicity of the compound, thus the LC-MS analysis time for a single run became longer, normally more than 25 minutes.

In the Li-method developed in Chapter I, auxiliary liquid was introduced after column separation. This encouraged us to apply the H-method to the other 3-keto steroids. Since the post-column modification will not change the retention time of each target, the steroids in my analysis will have their particular retention time if the auxiliary pump is off when analyzing the 3-keto steroids. In this case, multi-class steroids profiling could be achieved by the combination of the two methods within two analysis (26 minutes).

III.2. Material and Methods

III.2.1 Chemicals

Androstenedione (AE), adrenosterone (AT), estrone (E1), estradiol (E2) were supplied by from Tokyo Chemical Industry (Tokyo, Japan). 11-Deoxycorticosterone (DOC), 11-deoxycortisol (COS), 17α-hydroxyprogesterone (17-OH-P4), cortisone (COR), cortisol (COL), corticosterone (COB), dehydrotestosterone (DHT), progesterone (P4), testosterone (TE), corticosterone-9,11,11,12-d_4 (COB-d_4) and progesterone-2,3,4-$^{13}C_3$ (P4-$^{13}C_3$) were purchased from Sigma-Aldrich (Tokyo, Japan). Estradiol-d5 (E2-d_5) was supplied by Toronto Research Chemicals (North York, Canada).

The structure of the steroids was shown in Fig. III.1.

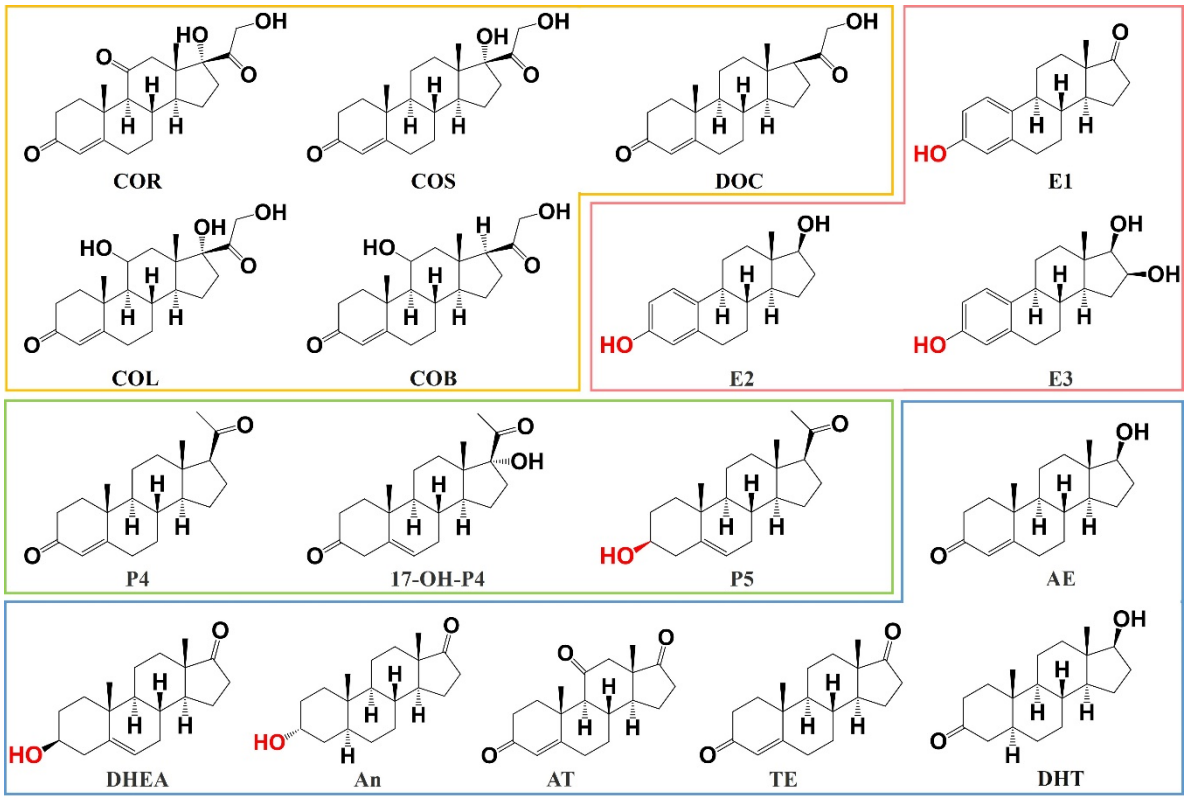


Figure III.1. Structures of the steroids used in this analysis. The boxes shown in yellow, pink, green, and blue represent different classes of steroid hormones, i.e. corticosteroids, estrogens, progestogens, and androgens, respectively

III.2.2 Preparation of standard stock, calibration, and quality control stock solutions

Each steroid standard was prepared in methanol at a concentration of 4 mg mL^{-1} as stock solutions and stored in -80 °C. These stock solutions were mixed and diluted to 10 ng μL^{-1} with 40% MeOH as mixed stock solutions at -80 °C. The working standard solutions were prepared at concentrations of 0.2, 0.4, 1, 2, 4, 10, 20, 40, 100, 200, 400, 1000 pg μL^{-1} with 40% MeOH. An

internal standard (IS) mixture solution of 100 pg μL^{-1} E3-d_3 and COB-d_4, and 40 pg μL^{-1} of P4-$^{13}C_3$ was prepared in 40% MeOH.

Blank matrix protocol was described in II.2.5, but with a slight modification. The eluate was evaporated to dryness with speed-vac and re-dissolved with 40% MeOH/H2O to form 10 mg μL^{-1} of blank matrix. For calibration and quality control samples, 6 µL of blank matrix was spiked with 6 µL of a working standard solution and 6 µL of IS solution. The samples were evaporated to dryness and re-dissolved in 12 µL of 40% MeOH. Finally, the calibration samples were at levels of 0.02, 0.04, 0.1, 0.2, 0.4, 1, 2, 4, 10, 20, 40, 100 pg mg^{-1}.

III.2.3 Animals and sample preparation

Animals and sample preparation protocol was described in Chapter II, but with a slight modification. Internal standards (6 µL of 1000 pg μL^{-1} E3-d_3 and COB-d_4, and 400 pg μL^{-1} of P4-$^{13}C_3$ in 40% MeOH) were added to each brain sample.

III.2.4 LC-MS/MS and other instruments

The UPLC-MS/MS analysis method was described in Chapter I (H method). The MRM parameters are summarized in Table III.1. Other Instruments used for sample preparation were described in I.2.3.

Table III.1. Parameters for the analysis of the steroids by LC-MS/MS. The auxiliary pump was off.

Analyte	Precursor ion form	RT/ min	Precursor Ion m/z	Fragmentor	Product Ion m/z	Collision Energy	IS
E3-d_3	$[M-H_2O+H]^+$	3.23	274.2	110	230.2 **135.1**	13 **25**	ISTD
E3	$[M-H_2O+H]^+$	3.25	271.2	110	159.1 **133.1**	25 **25**	E3-d_3
COR	$[M+H]^+$	3.88	361.2	140	121.1 **163.1**	37 **29**	COB-d_4
AT	$[M+H]^+$	3.98	301.2	140	121.1 **257.1**	29 **25**	COB-d_4

COL	$[M+H]^+$	4.24	363.2	140	**121** 105	**25** 55	COB-d_4
COB-d_4	$[M+H]^+$	5.32	351.2	110	**121.1** 97.1	**25** 33	ISTD
COB	$[M+H]^+$	5.34	347.2	110	105.1 **121.1**	53 **29**	COB-d_4
COS	$[M+H]^+$	5.37	347.2	140	109 **97**	33 **29**	COB-d_4
AE	$[M+H]^+$	5.94	287.2	140	**109** 97.1	**29** 25	COB-d_4
E1	$[M+H]^+$	6.07	271.2	140	133.1 **157**	25 **25**	COB-d_4
E2	$[M+H]^+$	6.19	273	100	**107** 135	**36** 20	COB-d_4
DOC	$[M+H]^+$	6.29	331.2	140	109 **97**	29 **29**	COB-d_4
TE	$[M+H]^+$	6.51	289.2	130	**109.1** 97.1	**29** 25	COB-d_4
DHEA	$[M-H_2O+H]^+$	6.86	271	130	**197.2** 159.2	**21** 2	COB-d_4
17-OH-P4	$[M+H]^+$	6.87	331.2	120	109.1 **97.1**	33 **29**	COB-d_4
DHT	$[M+H]^+$	7.48	291.2	110	105 **255**	49 **17**	P4-$^{13}C_3$
P4-$^{13}C_3$	$[M+H]^+$	7.98	318.2	130	**100.1** 112.1	**25** 25	ISTD
P4	$[M+H]^+$	8	315.2	130	109.1 **97.1**	29 **25**	P4-$^{13}C_3$
An	$[M-H_2O+H]^+$	8.35	273	140	199.1 **255.2**	21 **13**	P4-$^{13}C_3$
P5	$[M-H_2O+H]^+$	8.66	299	120	**159.1** 161.1	**25** 25	P4-$^{13}C_3$

The m/z values of product ions (MRM transitions) for each analyte in bold was used as the quantifying ion all other ions were used as qualifying ions.

III.3 Results and discussion

III.3.1 Chromatograms

The seventeen steroids could be detected with a lower background by the "H method" within nine minutes (Fig. III.2). The COS and COB are isobaric and eluted closely, giving the crossover peak as the shoulder peaks, although they could be separated at different retention times.

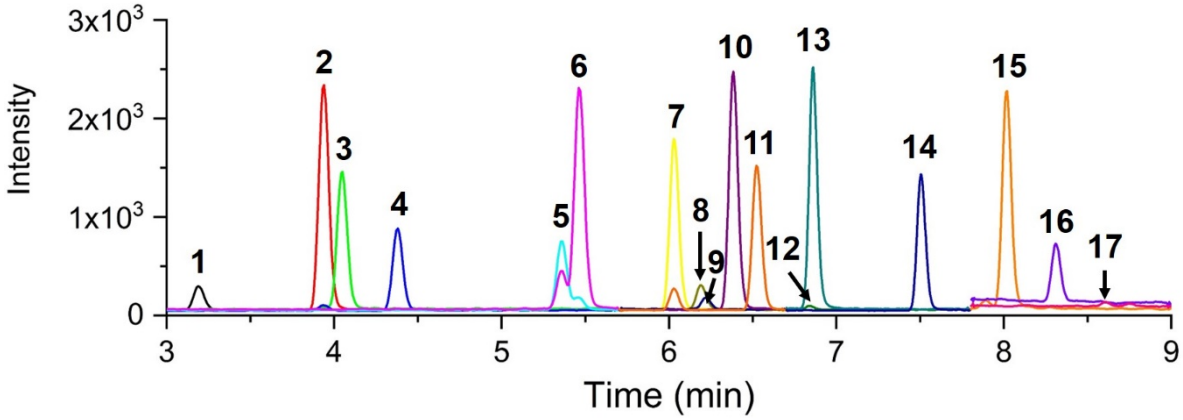


Figure III.2. MRM Chromatograms for the separation of steroids with 100 pg being injected. 1: E3, 2: COR, 3: AT, 4: COL, 5: COB, 6: COS, 7: AE, 8: E1, 9: E2, 10: DOC, 11: TE, 12: DHEA, 13:17-OH-P4, 14: DHT, 15: P4, 16:An, 17: P5. The MRM transitions used in this analysis were shown in Table III.1. The peak traces depicted in colors correspond to those obtained by the transitions indicated in bold, which were used for the quantification.

III.3.2 Method validation

The developed method was satisfactorily validated in terms of the limit of quantification (LOQ), linear range, extraction recovery, precision, and accuracy.

Calibration curves and LOQ

The calibration curves, correlation coefficients, linear ranges, and LOQs of the 13 steroids in the spiked blank matrix are shown in Table III.2. The calibration curve was constructed using the peak area ratios of a compound to IS versus the ratios of concentrations of a compound at different levels to the concentration of IS. The correlation coefficient square (r^2) was calculated. LOQ was tested at a signal to noise (S/N) of 10. Good linearity was observed for all 13 steroids within the

ranges (0.1-100 pg mg^{-1} for COR and DOC, 0.2-100 pg mg^{-1} for AT, COS, AE, 17-OH-P4 and DHT, 0.4-100 pg mg^{-1} for COL, COB, E1, TE and P4, 1-100 pg mg^{-1} for E3 and An, and 4-100 pg mg^{-1} for E2, DHEA and P5). The linear correlation coefficient square (r^2) was greater than 0.9990. The lower LOQ (LLOQ) was 0.035-3.433 pg mg^{-1} in mouse brain, suggesting that the developed method is highly sensitive for the quantification of those steroids.

Table III.2. Calibration curves, linearity range, and LOQs of steroids in a blank matrix obtained by the developed method.

NO.	Analyte	LOQ(pg/mg)	Slope	Intercept	r^2	Linearity range(pg/mg)
1	E3	0.609	1.735	-0.074	0.9958	1-100
2	COR	0.076	8.344	0.034	0.9994	0.1-100
3	AT	0.084	5.292	-0.064	0.9992	0.2-100
4	COL	0.210	2.706	0.106	0.9991	0.4-100
5	COB	0.165	2.906	0.106	0.9991	0.4-100
6	COS	0.093	9.410	-0.178	0.9992	0.2-100
7	AE	0.087	6.448	-0.204	0.9991	0.2-100
8	E1	0.264	0.791	-0.015	0.9994	0.4-100
9	E2	1.376	0.358	-0.068	0.9953	4-100
10	DOC	0.035	8.72	-0.088	0.9994	0.1-100
11	TE	0.275	5.313	-0.183	0.9993	0.4-100
12	DHEA	3.433	0.113	-0.015	0.9947	4-100
13	17-OH-P4	0.147	8.667	-0.312	0.9990	0.2-100
14	DHT	0.132	1.947	-0.212	0.9990	0.2-100
15	P4	0.213	5.007	-0.697	0.9993	0.4-100
16	An	0.526	0.830	-0.294	0.9930	1-100
17	P5	2.474	0.046	-0.005	0.9955	4-100

Matrix effect and recovery

To evaluate the matrix effect (ME) and recovery (R), low (0.2 or 4 pg mg^{-1}), medium (4 or 20 pg mg^{-1}) and high (20 or 40 pg mg^{-1}) concentrations of spiked blank matrix and mouse brain samples (n = 3) were assessed. The matrix effect value was calculated as ME (%) = $A_{matrix}/A_{solution} \times 100$, where $A_{solution}$ is the compound peak area of 10 μL of pure standard and A_{matrix} is the compound peak area of blank matrix spiked with 10 μL standard sample. The recovery value was

calculated as R (%) = $A_{pre\text{-}spike}/A_{post\text{-}spike} \times 100\%$, where $A_{pre\text{-}spike}$ is the compound peak area of mouse brain spiked with 10 μL standards before extraction and $A_{post\text{-}spike}$ is the compound peak area of brain sample spiked with 10 μL standards after extraction. The matrix effect and extraction recoveries (%) were within the range of 88.75-113.98% and 79.35-117.66% in Table III.3.

Table III.3. Matrix effect and recovery of 13 steroids in mouse brain.

No.	Analyte	spiked conc. (pg/mg)	matrix effect%		recovery%	
			mean%	RSD %	mean%	RSD %
1	E3	4.00	100.43	10.09	81.94	10.10
		20.00	105.72	6.00	79.35	6.52
		40.00	107.98	4.56	117.66	9.98
2	COR	0.20	100.61	0.87	93.33	0.78
		4.00	104.63	7.86	93.75	3.73
		20.00	108.73	5.70	108.22	3.74
3	AT	0.20	99.80	1.11	101.84	4.72
		4.00	104.12	9.13	93.81	3.66
		20.00	110.84	5.97	108.68	4.38
4	COL	4.00	104.44	6.96	92.97	3.08
		20.00	109.61	5.18	106.07	5.64
		40.00	107.24	7.69	97.76	2.17
5	COB	4.00	108.55	6.00	99.39	3.76
		20.00	108.64	4.82	112.76	3.76
		40.00	106.41	7.46	103.55	0.24
6	COS	4.00	103.38	7.32	95.36	2.98
		20.00	108.13	5.79	108.91	3.92
		40.00	107.04	7.64	99.85	2.48
7	AE	0.20	100.28	1.51	102.86	11.66
		4.00	102.41	10.40	95.46	3.29
		20.00	107.00	5.83	110.11	2.33
8	E1	4.00	100.36	9.45	88.88	5.51
		20.00	97.38	5.66	102.29	1.80

		40.00	93.94	6.81	93.12	2.28
		4.00	103.62	5.92	93.96	8.26
9	E2	20.00	99.39	7.88	103.05	4.65
		40.00	92.52	7.23	90.39	0.89
		4.00	103.45	9.85	95.10	3.13
10	DOC	20.00	106.33	5.88	109.81	3.84
		40.00	103.97	7.51	101.28	2.44
		4.00	101.03	8.16	97.89	5.84
11	TE	20.00	105.65	6.40	111.74	3.48
		40.00	102.89	7.99	100.88	2.94
		4.00	103.39	9.74	94.73	6.31
12	DHEA	20.00	105.25	11.05	109.70	11.71
		40.00	98.23	7.47	94.68	7.54
		4.00	104.27	10.00	90.12	2.33
13	17-OH-P4	20.00	109.21	5.34	111.33	3.15
		40.00	107.06	7.36	101.22	0.35
		4.00	106.01	1.98	87.38	4.86
14	DHT	20.00	108.60	9.89	89.83	2.10
		40.00	105.84	9.46	86.24	1.67
		4.00	102.40	6.61	87.41	4.03
15	P4	20.00	107.68	6.49	91.49	3.70
		40.00	105.54	5.72	89.48	2.90
		4.00	108.46	2.55	89.94	6.31
16	An	20.00	106.97	5.39	93.67	5.76
		40.00	93.20	10.15	88.43	2.22
		4.00	110.93	7.73	107.09	4.40
17	P5	20.00	113.98	4.52	92.12	10.94
		40.00	88.75	5.56	79.92	1.30

Accuracy and precision

The precision and accuracy of the method were assessed by performing six replicates of mouse brain samples spiked with low (0.2 or 4 pg mg^{-1}), medium (4 or 20 pg mg^{-1}) and high concentrations (20 or 40 pg mg^{-1}) of steroids (Table III.4). Accuracy was calculated as the averaged percentage for the measured concentrations to the real concentrations. Precision was

expressed as the RSDs (relative standard deviation) of the measured concentrations and was performed on three separate days. The low and high concentration of steroids spiked in the intra and inter-batch samples were obtained within acceptable ranges. Table III.4 summarizes the accuracy and precision data for mouse brain. The results indicated that the method shows a moderately good precision and accuracy. The accuracy (%) was 77.56-117.74 while the intra-day precision (%) was 0.35-18.73 and the Inter-day precision (%) was 1.05-18.03. The method was considered to be suitable in terms of accuracy and precision.

Table III.4. Accuracy and intra-day and inter-day precision in the analysis of 13 steroids in mouse brain.

No.	Analyte	spiked conc. (pg/mg)	Mean (pg/mg)	Accuracy(%)	Intra-day precision(%)	Inter-day precision(%)
		4	3.48	86.92	7.77	8.98
1	E3	20	15.51	77.56	7.99	7.61
		40	33.12	82.79	7.63	5.36
		0.2	0.23	114.30	1.61	4.89
2	COR	4	3.37	84.16	5.35	8.16
		20	17.60	87.99	2.63	1.28
		0.2	0.24	117.74	4.99	8.26
3	AT	4	3.47	86.86	1.56	4.88
		20	16.56	82.79	3.40	1.50
		4	3.59	89.82	2.48	6.79
4	COL	20	18.13	90.64	5.72	2.83
		40	34.30	85.75	3.87	5.98
	COB	4	4.18	104.44	11.03	13.08
5		20	17.91	89.56	12.72	8.63
		40	32.81	82.03	0.75	7.56
		4	3.52	87.93	9.70	8.37
6	COS	20	18.18	90.91	3.98	1.05
		40	33.52	83.80	2.38	6.40
		0.2	0.18	89.56	0.64	11.09
7	AE	4	4.18	104.46	9.22	4.62
		20	18.16	90.79	2.38	5.26

		4	4.29	107.15	9.75	10.48
8	E1	20	19.28	96.42	4.81	18.03
		40	39.91	99.79	14.04	2.35
		4	4.55	113.73	11.74	1.43
9	E2	20	20.56	102.80	3.26	13.28
		40	43.09	107.71	8.62	8.62
		4	4.17	104.21	4.03	8.52
10	DOC	20	19.70	98.48	6.19	5.63
		40	42.75	106.89	6.68	2.46
		4	4.14	103.38	12.35	10.85
11	TE	20	18.28	91.42	3.78	3.20
		40	43.58	108.95	4.57	8.90
		4	4.25	106.23	15.29	2.08
12	DHEA	20	19.42	97.09	13.47	10.32
		40	33.76	84.39	9.86	3.81
		4	3.69	92.28	2.33	3.23
13	17-OH-P4	20	17.82	89.08	3.24	7.86
		40	33.05	82.62	0.35	2.45
		4	4.05	101.21	4.86	7.77
14	DHT	20	18.30	91.48	5.87	5.22
		40	35.90	89.75	6.65	9.39
		4	3.77	94.28	6.92	13.12
15	P4	20	17.70	88.52	4.25	7.74
		40	34.26	85.65	6.18	4.07
		4	3.72	93.02	8.00	11.05
16	An	20	19.71	98.53	4.59	10.02
		40	35.97	89.93	4.63	3.39
		4	4.39	109.87	18.73	5.95
17	P5	20	18.60	93.01	1.02	4.58
		40	42.42	106.04	15.12	12.84

III.3.3 Biosample analysis

The method was applied for the analysis of steroids in brain tissue from six healthy male mice. Nine steroids were identified and quantified in whole brain samples. The steroids were identified by comparing the retention time and the peak intensity ratio of two MRM transitions of the samples with those shown for a spiked blank matrix sample. The criteria for the identification was as follows: the relative difference in retention time should be less than 2% the relative difference in the ratio of the peak intensity of two MRM transitions should be less than 20%. The method allowed nine steroids to be identified from one mouse brain tissue sample (Fig. III.3). The shoulder peak for COB on the right side in the spiked blank matrix sample is the peak derived from the spiked COS. Note that there are shoulders to the DHEA peaks on the left sides, which were, most probably, due to the endogenous metabolites in the brain extract that were slightly observed for the blank matrix sample.

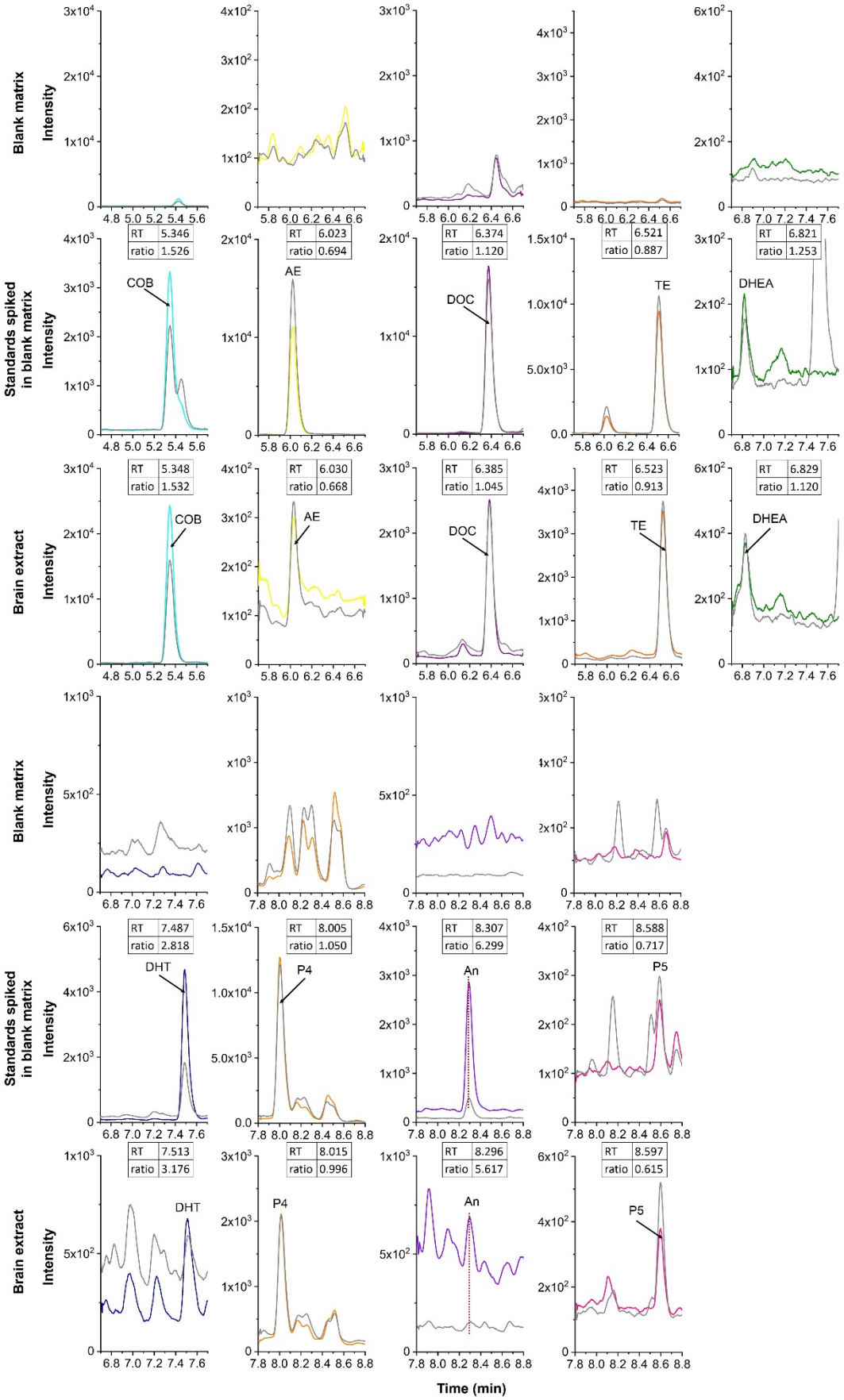

Blank matrix
Intensity
Standards spiked in blank matrix
Intensity
Brain extract
Intensity
Blank matrix
Intensity
Standards spiked in blank matrix
Intensity
Brain extract
Intensity
RT 5.346
ratio 1.526
COB
RT 6.023
ratio 0.694
AE
RT 6.374
ratio 1.120
DOC
RT 6.521
ratio 0.887
TE
RT 6.821
ratio 1.253
DHEA
RT 5.348
ratio 1.532
COB
RT 6.030
ratio 0.668
AE
RT 6.385
ratio 1.045
DOC
RT 6.523
ratio 0.913
TE
RT 6.829
ratio 1.120
DHEA
RT 7.487
ratio 2.818
DHT
RT 8.005
ratio 1.050
P4
RT 8.307
ratio 6.299
An
RT 8.588
ratio 0.717
P5
RT 7.513
ratio 3.176
DHT
RT 8.015
ratio 0.996
P4
RT 8.296
ratio 5.617
An
RT 8.597
ratio 0.615
P5
Time (min)

Figure III.3. MRM chromatograms of steroids from a charcoal-treated spiked sample (upper panels for each) and a whole mouse brain (lower panels). Each steroid was identified using two MRM transitions (Table III.1). The colored traces in each chromatogram are depicted for the boldface values (=MRM transitions) in Table III.1, which were used for the quantification. The gray traces represent another transitions (normal values in Table III.1), which were used for the qualification. The amounts of steroids spiked to the charcoal treated sample was 500 pg for each. The ratios indicated in the insets were calculated on the basis of the peak height ratios of the two transitions (normal over boldface values for each steroid in Table III.1).

As shown in Table III.5, the amount of steroids in the tissue varied from sample to sample, ranging from 0.10 to >80 pg mg^{-1}. In earlier studies, all these steroids were quantified in whole rat brain at concentrations of 0.1-58 pg mg^{-1}. The level of COB, AE, DOC, TE, DHT An and P4 in the mouse brain is similar to those in the rat brain. However, in this analysis, the level of DHEA was found to be still much higher in the mouse brain tissue. The results in Chapter II, obtained using Li-method, showed similar result (see Table II.5 and III.5). It could be concluded that the relatively high level of DHEA could be ascribable to the mice species used in the present experiment. The level of P5 detected by Li-method was 1.64-1.98 (Table II.5) while the LOQ of P5 in H-method was 2.474 pg mg^{-1} (Table III.5). This is the reason that we could not get the accurate level of P5.

Table III.5. Quantitative results for the steroids of whole mouse brain tissue and a comparison with the values reported in previous studies.

Steroid	concentration in whole brain (pg mg^{-1}), n = 6 [a]	Literature concentration in male rat brain(pg mg^{-1})
COB	46.84 - >80	<12.1[10] 22-58[44]
AE	0.10 - 0.21	0.1[34]
DOC	0.34 - 2.10	0.1[34]
TE	0.23 - 2.12	0.11-0.48[60] 1.4 - 2.2[1]
DHEA	8.07 - 10.48	0.05-0.11 [60] 0.2-0.6 [63]
DHT	0.45 - 0.82	0.16-1.25[63]

P4	1.04 - 4.01	0.5-3[34] 21 - 47[44]
An	0.60 - 1.10	0.18 ± 0.10[66]
P5	<LOQ	0.60-1.2 [60], 7 [62]

LOQ: limit of quantitation.

[a] The concentration in each individual brain is shown in Table III.6.

Table III.6. Quantitative results for the steroids detected in each mouse brain tissue (n = 6).

Steroid	Concentration in whole brain (pg mg^{-1}), n = 6					
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6
COB	59.45±0.77	77.98±0.80	>LOQ	>LOQ	>LOQ	46.84±0.48
AE	0.21±0.04	0.10±0.01	0.14±0.02	0.19±0.02	0.20±0.02	0.18±0.02
DOC	0.84**±0.04**	0.91±0.03	1.22±0.04	2.10±0.07	1.38±0.03	0.34±0.02
TE	2.12±0.10	0.23±0.01	0.33±0.01	0.60±0.02	0.50±0.01	0.37±0.01
DHEA	10.48±0.29	8.07±0.26	9.89±0.86	9.83±0.55	10.03±0.44	9.84±0.71
DHT	0.82±0.05	0.46±0.01	0.56±0.02	0.58±0.03	0.65±0.01	0.68±0.07
P4	1.04±0.03	2.00±0.14	2.71±0.13	4.01±0.16	3.58±0.60	2.52±0.48
An	0.80±0.06	0.63±0.04	0.60±0.03	0.74±0.04	0.98±0.07	1.10±0.11

III.4 Conclusion

It is the first report on the profiling of twenty-eight steroids by the combination of Li-method and H-method. Thirteen steroids were detected in whole mouse brain although the actual role of these steroids in the brain needs to be elucidated and will require further investigation. The method has a high potential for allowing the determination of various steroids in separate regions of a brain tissue such as the cerebrum, cerebellum, hippocampus, and hypothalamus.

General Conclusion

In this thesis, lithium ion adduction-based LC-MS/MS analysis of multi-class ketolic steroid hormones containing a 3-OH group was established. I found that the adduction of Li^+ to molecular ions significantly depressed the dehydration of 3-OH steroids during measurement. The method could be successfully applied for profiling the steroids in a mouse brain tissue to reveal three new steroids that have not been reported so far. The results obtained are as follows:

1) The novel method utilizing Li^+ as an adduct in ESI-MS ("Li-method") was developed. It allowed for the sensitive and universal quantitation of multi-class 3-OH steroids. By the post-column addition of Li ions into the LC mobile phase, the sensitivity and selectivity could be remarkably enhanced by 1.53-188 times for 13 different types of 3-OH steroids over the "H-method". This also represents the first published method describing the analysis of lithiated steroids by LC-MS/MS. **(Chapter I)**
2) The 13 steroids, whose detection sensitivities were enhanced by the Li-method, contain, at least, one keto and two hydroxyl groups or one keto and 5-olefinic double bond. **(Chapter I)**
3) The optimization of sample pretreatment procedure for isolating free steroids from a mouse brain tissue and the above Li-method allowed six steroids to be detected from one mouse brain tissue, among which 16-OH-E1, THB, and 17-OH-P5 were firstly found in a control mouse brain. **(Chapter II)**
4) The H-method had a higher sensitivity for An, E3, and 3-keto steroids. In addition, since the analytical settings for the H- and Li-methods are quite similar, they could be simultaneously operated in the same system, or achieved separately within two analyses (26 min). The combined use of both methods allowed profiling of twenty-eight steroids. **(Chapter III)**
5) By using a combination of H- and Li-methods, thirteen steroids, including the above 16-OH-E1, THB, and 17-OH-P5, could be identified in one mouse brain tissue sample. **(Chapter III)**

The findings in this thesis strongly suggest that the combined use of H- and Li-methods could allow for profiling of steroids in quite limited amounts of biosamples such as the individual tissues of a brain, e.g., cerebrum, cerebellum, hippocampus, hypothalamus, etc. which should give insights into their functions relative to physiological conditions or responses.

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When I am preparing for this thesis, my home country is still suffering from the coronavirus outbreak. I predicted the outbreak one week before it came true thanks for my research field and interest. My family was informed once I knew the situation and they are still healthy. I wish this plague would come to the end soon.

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List of publication

1. **Qiuyi. Wang**, Kimiko. Shimizu, Kanako. Maehata, Yue. Pan, Koki Sakurai, Takatoshi. Hikida, Yoshitaka. Fukada, and Toshifumi. Takao, Lithium ion adduction enables UPLC-MS/MS–based analysis of multi-class, 3-hydroxyl group–containing keto-steroids, Journal of lipid research, in press (doi:10.1194/jlr.D119000588)

Related papers

1. Yun-Qing Huang[1], **Qiu-Yi Wang**[1], Jia-Qi Liu, Yan-Hong Hao, Bi-Feng Yuan and Yu-Qi Feng. Isotope labelling-paired homologous double neutral loss scan-mass spectrometry for profiling of metabolites with a carboxyl group. Analyst 2014, 139, 3446–3454

2. Bao-Ling Qi[1], Ping Liu[1], **Qiu-Yi Wang**, Wen-Jing Cai, Bi-Feng Yuan, Yu-Qi Feng. Derivatization for liquid chromatography-mass spectrometry. Trends in Analytical Chemistry 2014, 59: 121-132.

3. **Qiu-Yi Wang**, Tiantian Ye, Shu-Jian Zheng, Er-Cui Ye, Ren-Qi Wang and Yu-Qi Feng, A stable isotope labelling assisted LC-MS method for the determination of polyamines in micro-tissues of rice, Analytical Methods, 2017, 9, 3541-3548

4. Kanako Maehata, Kimiko Shimizu, Tomoko Ikeno, **Qiuyi Wang**, Toshifumi Takao and Yoshitaka Fukada, 7α-OH-pregnenolone and 7α-OH-DHEA in mouse hippocampus bolster remote spatial memory, submitted to journal