Review

Trace Determination of Steroids Causing Age-Related Diseases Using LC/MS Combined with Detection-Oriented Derivatization

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Received July 31, 2006

With the rapid shift to an aging society in Japan, age-related diseases, such as osteoporosis, dementia and cancer, are sharply increasing. The measurement of steroids related to these diseases in biological fluids and tissues is useful for elucidation of the nature, diagnosis and treatment of such diseases. LC/MS is considered to be the most promising method for this purpose due to its specificity and versatility, but it sometimes does not demonstrate the required sensitivity for trace amounts of steroids, because steroids have a rather low response using either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). To overcome this problem, the author developed detection-oriented derivatization procedures for steroids in LC/MS. For ESI-MS, introduction of a permanently charged moiety is effective. Based on this, 2-hydrazino-1-methylpyridine was developed and used in monitoring prostatic 5α-dihydrotestosterone, a good index for the follow-up of patients affected by prostate cancer under androgen deprivation therapy and salivary dehydroepiandrosterone, which is now often designated as an anti-aging hormone. A proton-affinitive Cookson-type reagent, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione, was used for the determination of 1α-hydroxyvitamin D_3 [1α(OH)D_3], a synthetic prodrug of the active form of vitamin D_3, in human plasma, and this new LC/positive-APCI-MS method enabled the pharmacokinetic study of 1α(OH)D_3 in humans. Electron-capture APCI-MS based on derivatization with 2-nitro-4-trifluoromethylphenylhydrazine was used for the analysis of neurosteroids, which affect brain excitability through action at the neurotransmitter receptors. With this method, the stress-induced rapid biosynthesis of pregnane-type neurosteroids in rat brains was demonstrated.

Key words LC/MS; steroid; detection-oriented derivatization; age-related disease

1. Introduction

Classical steroid hormones (estrogens, androgens, gestogens and corticoids) are biomolecules which mostly act as ligands to intracellular or nuclear receptors. Their precursors also play an important role in humans. It is well known that an active metabolite of vitamin D_3 (9,10-secosteroid), 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], also has hormonal properties (calcium homeostasis, bone formation and so on) *via* binding to its specific nuclear receptor. On the other hand, some oxosteroids have been found to be modulators of several membrane receptors in the nervous system, and such steroids are now universally referred to as neurosteroids (NSs).[1] These steroids exert strong biological activities at very low concentrations (nanomolar and even picomolar) in the target tissues, and some of the natural steroids and numerous synthetic steroids have been used as therapeutic agents.

With the rapid shift to an aging society in Japan, age-related diseases, such as osteoporosis, dementia and cancer, are sharply increasing. A specific and sensitive method for the characterization and determination of steroids related to these diseases in biological fluids and tissues is useful for the elucidation of the nature, diagnosis and treatment of the diseases. Because of the close structural similarity, the metabolic versatility and their occurrence at low concentrations in body fluids and tissues, the development of reliable analytical methods of the steroids is a challenging subject for analytical chemists. Numerous methods have been described to characterize and determine the steroids, such as immunoassay, receptor binding assay, HPLC and GC/MS,[2,3] but every one of these approaches has both advantages and disadvantages.

LC coupled with atmospheric pressure ionization (API), such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)-MS is considered to be the most promising method for the steroid analysis due to its specificity and versatility. However, the ionization efficiencies of most of the steroids are relatively low for ESI and APCI. Therefore, conventional LC/MS sometimes does not demonstrate the required sensitivity for the trace analysis of steroids. To overcome this, the author developed detection-oriented derivatization procedures for the LC/MS analysis of steroids. This review describes the author's recent studies on the development and application of the derivatization–LC/MS method for the trace determination of steroids related to age-related diseases.

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2. Is Derivatization Necessary in LC/MS?

In LC/MS assays, it goes without saying that it is important to select the ionization methods (ESI or APCI) and ionization polarity (positive-ion or negative-ion mode) suitable for the analyte. Instrument parameters, such as the flow rate of the nebulizer gas and desolvent gas, desolvation temperature and electrospray capillary voltage, can significantly affect the generation of ions in LC/API-MS detection, and therefore, the optimization of these parameters is a necessary part of the development of analytical methods. The use of the selected reaction monitoring (SRM) mode may allow for the improvement of the assay sensitivity, because it can significantly reduce background noise derived from matrices. However, the chemical and physical properties of the analyte are the most critical parameters for superior sensitivity in the various modes of ionization. Although the introduction of a newly developed API technique, atmospheric pressure photoionization (APPI),[4,5] and application of mobile phase additives[6,7] have also been examined for low or middle polarity analytes, including steroids, the author believes that derivatization is the most effective and reliable method for increasing the sensitivity of the target analyte. That is, derivatization changes the chemical and physical properties of the analyte, resulting in its ionization efficiency (conversion of a poorly ionizable compound into a derivative that is easily detectable by API-MS). The chromatographic behavior of the analyte will also be changed after derivatization, therefore, decreasing the suppression of ionization related to the co-elution of biomatrix components. That is, derivatization increases not only the sensitivity, but also the specificity.

The author is well aware that the most common advantage of LC/MS over GC/MS is the elimination of a derivatization step. However, this advantage is not the only advantage. When a derivatization step is necessary for both GC/MS and LC/MS, often the sample handling step and the instrument operation and maintenance for LC/MS are easier than for GC/MS. Also, the chromatographic run time for LC/MS is generally shorter than that of GC/MS. Furthermore, many different kinds of analytical columns (separation modes) are available for LC, which are effective for the separation of an analyte and interferences.

The derivatization reagents for LC/MS consist of the reacting group for the respective functional groups and the responsive moieties for API-MS. Because steroids usually have hydroxy and/or keto groups, these functional groups can be used to introduce the API responsive moieties. The *s-cis*-diene of the vitamin D compounds is also usable for derivatization. In this article, a derivatization reagent for LC/MS is called the "MS tag."

3. Instruments

In the author's studies presented in this article, an Applied Biosystems API 2000 triple stage quadrupole-mass spectrometer (Foster City, CA, U.S.A.) and a ThermoQuest LCQ ion trap-mass spectrometer (San Jose, CA, U.S.A.) were used in the ESI and APCI modes, respectively.

4. MS Tag for Positive ESI

ESI has become an important API technique for the generation of gas-phase ions from biomolecules for MS analysis. Because the ionization process occurs in the solution-phase during ESI, the best detectability with ESI-MS has been achieved for the analysis of analytes either that are ionic or that can be readily ionized in solution. In other words, the pre-formation of ions is very important in the ESI detection mode. Most steroids have no ionic or easily ionizable moiety, and therefore, their ESI sensitivities are extremely poor; the application of ESI-MS to the steroid analysis has been limited. The author's idea to overcome this problem is the introduction of permanently charged moieties to the target analyte, that is, "charged derivatization."

One of the useful permanently charged moieties in the positive ESI-MS is the *N*-alkylpyridyl group. Girard reagent P (GP) is an already-known derivatization reagent for carbonyl compounds to form hydrazones with a quaternary pyridinium moiety, and its application for the LC/ESI-MS of steroids has lately attracted considerable attention. Griffiths *et al.*[8] reported that the GP derivatives of some 3-oxo-4-ene-steroids, such as testosterone (T), could be detected and identified at the sub-picograms level by ESI-MS. However, oxo-groups at the 17- and 20-positions were less reactive than that at the 3-position, and therefore, the products of the reaction with GP were a mixture of the mono- and bis-hydrazones in the derivatization of androstenedione and progesterone (PROG). Thus, the *N*-alkylpyridylation of some steroids has a significant advantage for increasing their de-

Tatsuya Higashi was born in 1968 in Ishikawa Prefecture, Japan. He graduated with a B.S. degree in Pharmaceutical Sciences from Kanazawa University in 1991. He received his M.S. degree from the same university under the direction of Professor Kazutake Shimada and Dr. Norihiro Kobayashi (now, Professor of Kobe Pharmaceutical University) in 1993. After he was engaged in the preclinical and phase I studies of drug development at Tsukuba Research Laboratories of Nippon Glaxo K. K. (1993—1995), he returned to Professor Shimada's research group as a Research Assistant (1995) and worked as a Research Associate from 1996. In 1999, he obtained his Ph.D. from Kanazawa University working on the development of the enzyme immunoassay of 24,25-dihydroxyvitamin D_3. He was promoted to an Assistant Professor in 2001 and then an Associate Professor in 2003. He received the Japan Society for Analytical Chemistry Award for Young Scientists in 2003 and the Pharmaceutical Society of Japan Award for Young Scientists in 2006. His current interests are the development of methods for increasing the sensitivity in LC/MS to detect and characterize trace amounts of biologically active steroids.

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tection responses in ESI-MS, but GP is not always satisfactory for practical applications. Based on this background information, the author designed and synthesized 2-hydrazino-1-methylpyridine (HMP) as a new derivatization reagent with a quaternary pyridinium moiety for oxosteroids (Fig. 1).[9] HMP rapidly (15—60 min) and quantitatively reacted with 3-oxo-4-ene- (ex. T), saturated 3-oxo- [ex. 5α-dihydrotestosterone (DHT)], 17-oxo- [ex. dehydroepiandrosterone (DHEA)] and 20-oxo-steroids [ex. pregnenolone (PREG)] under mild conditions (room temperature or 60 °C) in the presence of an acid catalyst (trifluoroacetic acid). All the HMP derivatives provided only their molecular cation, $[M]^+$, and a 70—1600-fold higher sensitivity compared to the intact steroids (Table 1). The HMP derivatives were superior to the GP derivatives not only in their sensitivity [limit of detection (LOD): equal–10 times], but also in their chromatographic behavior; the GP derivatives showed a significant peak tailing, which is a major concern for the separation of the measuring steroids from endogenous interfering substances in biological samples, thus leading to a low assay accuracy and precision. On the contrary, the HMP derivatives gave satisfactorily shaped peaks.

This derivatization procedure was successfully applied to the determination of prostatic DHT in 10-mg of tissue.[10] Prostate cancer (PCa) is the most common malignancy in aged males in the United States and is also sharply increasing in Japan. Because PCa depends initially on androgens, primarily DHT, androgen deprivation therapies [ADT, usually combination of luteinizing hormone-releasing hormone (LH-RH) agonists and anti-androgens] are often first choice of several therapeutic procedures for PCa. For the evaluation of the effects of the hormone therapy in the clinical field, a highly sensitive method that enables the measurement of prostatic DHT using a small volume of sample (*ca.* 10 mg) collected by a biopsy needle is required. The HMP-derivatization increased the detection response of DHT by 130-times and the limit of quantitation (LOQ) of the prostatic DHT was 1.0 ng/g tissue for a 10-mg tissue aliquot. The method employed the SRM mode, in which the residual $[M]^+$ was used as the monitoring ion after the collision of $[M]^+$ with 30 eV. By this technique, the noise ions derived from the endogenous components were reduced without considerably decreasing the intensity of $[M]^+$ of the analyte. This method required only one solid-phase extraction (SPE) step for the purification of the prostatic sample, which is much simpler than the previously reported method.[11] The level of DHT in the benign prostatic hyperplasia (BPH) tissues (without ADT) was 5.18±1.32 ng/g tissue (mean±S.D., n=7) (Fig. 2). On the contrary, in all the PCa tissues (n=7), DHT was not detected, which is attributable to ADT. Thus, the present method was useful for the evaluation of the effects of the hormone therapy in PCa.

The HMP-derivatization was also used for the DHEA measurement. Apart from serving as a precursor of estrogens and androgens, DHEA has been considered to have no obvious biological function. However, recent studies suggest that DHEA is involved in the prevention of diseases that frequently develop in the aged. The administration of large doses of DHEA to rodents has demonstrated a multitude of beneficial effects on the prevention of cancer, heart diseases, diabetes and obesity.[12] In humans, the age-related decline in

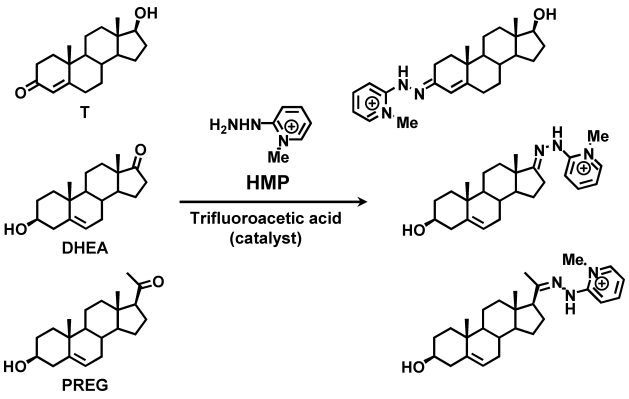

Fig. 1. Charged Derivatization of Oxosteroids with HMP

Table 1. LODs of Oxosteroids and Their HMP and GP Derivatives

Compound	LOD (fmol, S/N=5)	Relative detectability
T (intact)[a]	69.4 (20 pg)[b]	1
T-HMP	1.0 (0.3 pg)	70
T-GP	10.4 (3.0 pg)	7
DHEA (intact)	2780 (800 pg)	1
DHEA-HMP	1.7 (0.5 pg)	1600
DHEA-GP	4.2 (1.2 pg)	670
PREG (intact)	791 (250 pg)	1
PREG-HMP	1.6 (0.5 pg)	500
PREG-GP	1.6 (0.5 pg)	500

a) LODs of intact oxosteroids were determined using positive-APCI-MS. *b*) The values in parentheses are amounts converted into intact steroids.

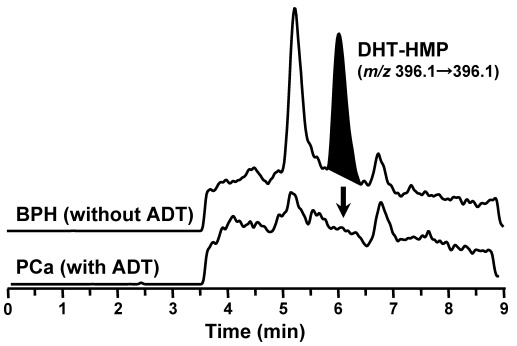

Fig. 2. Analysis of the Effect of ADT on the Prostatic DHT Level Using LC/ESI-MS/MS

the DHEA levels seems to be associated with depression, osteoporosis, autoimmune disease and the metabolic syndrome.[13] Although it is now unclear that these effects are due to DHEA itself or its downstream conversion products (estrogens and androgens), DHEA is now often designated as an anti-aging hormone and taken as a food supplement in the U.S.A. The serum or plasma specimen is conventionally used to measure individual DHEA levels in humans. On the contrary, saliva has recently been attracting attention as a new tool in clinical examinations and therapeutic drug monitoring due to its easy non-invasive nature of collection.[14] The use of saliva in steroid assay has an another advantage; the levels of steroids in saliva generally reflect those of the unbound steroids with protein (*i.e.*, bioavailable steroids) in serum/plasma. However, a major disadvantage in the use of saliva is its low analyte concentration; for example, the quan-

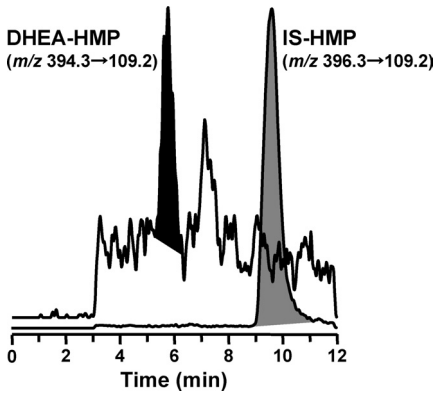

Fig. 3. Determination of the Salivary DHEA (25 pg/ml) as Its HMP Derivative Using LC/ESI-MS/MS

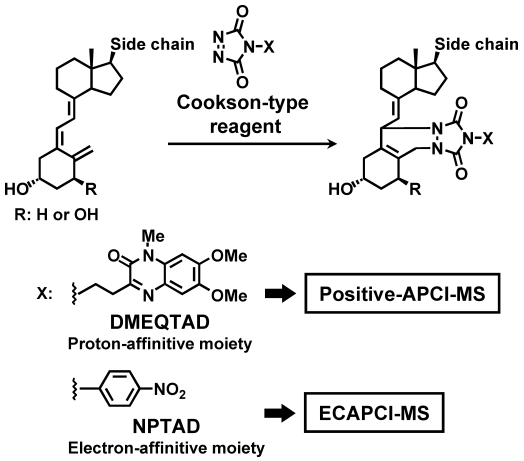

Fig. 4. Strategy for Increasing Detectability of Vitamin D_3 Compounds in LC/APCI-MS Based on Derivatization with Cookson-Type Reagents

tity of cortisol in saliva is 5—10 times lower than that of the total cortisol in serum.[15] With this background information, the author has developed the LC/ESI-MS/MS method combined with the HMP-derivatization for the determination of DHEA in human saliva.[16] The derivatization significantly increased the detectability of DHEA in the positive-ESI-MS (1600-fold), and the LOQ of the salivary DHEA was 25 pg/ml for a 200-μl sample aliquot (Fig. 3). The SRM mode using $[M]^+$ as the precursor ion and [*N*-methylpyridine$+NH_2]^+$ (cleavage of N–N bond of the hydrazone) was used for quantification. When the calibration curve was constructed using androsterone as the IS, a good linearity was obtained in the range of 5—200 pg per tube. This method was able to detect the diurnal rhythm and the age-related decline in the salivary DHEA, and was also applicable for the determination of the change in the individual DHEA levels after the DHEA supplementation. Thus, using this highly sensitive method based on the derivatization–LC/MS, the author succeeded in obtaining the biological information on DHEA from saliva, which had hitherto been obtained only from serum/plasma analyses.

5. MS Tag for Positive APCI

APCI is also widely used as an API technique. In contrast to ESI, in which the ionization process occurs in the solution-phase, APCI is a gas-phase ionization process. Ions generated by the usual APCI fundamentally result from the gain or loss of a proton, in which corona discharge initially generates a charged plasma from the ambient source atmosphere and evaporated solvent, and analytes passing though this plasma are then ionized through chemical charge transfer similar to that of chemical ionization (ion-molecular reaction). For weakly polar and neutral molecules, such as steroids, APCI is often the ionization source of choice. Steroids with the 3-oxo-4-ene-structure show relatively higher responses (LOD: $<$50 pg) in the positive-APCI-MS, because their protonated forms are stabilized due to the delocalization of the charge, *i.e.*, resonance stabilization.[17] However, the derivatization of steroids is still required for increasing their detection response in the APCI-MS mode, in consideration of their very low concentrations in body fluids and tissues and the limited sample volume.

In the positive-APCI-MS, the introduction of proton-affinitive moieties to the analyte is generally effective for increasing the sensitivity of the resulting derivatives. Because APCI is a gas-phase ionization process as mentioned above, contrary to the positive-ESI-MS, the introduction of oxygen and nitrogen atoms without an increase in the hydrophilicity (polarity) of the analyte is effective in the positive-APCI-MS; the introduction of a hydrophilic functional group sometimes decreases the sensitivity. One of the simplest derivatization procedures in the positive-APCI-MS is acetylation of the hydroxy groups of steroids.

The Cookson-type reagent is a 4-substituted 1,2,4-triazoline-3,5-dione (TAD), and TAD is a powerful dienophile which rapidly and quantitatively reacts with the *s-cis*-diene of the vitamin D compound to form a Diels–Alder adduct (Fig. 4). The author examined the application of Cookson-type reagents for the quantitative analysis of vitamin D compounds, which is useful for the diagnosis and treatment of bone diseases, such as osteoporosis and renal osteodystrophy.[18—20] The author first chose 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-TAD (MBOTAD) as the oxygen and nitrogen-rich Cookson-type reagent,[18] but later found that 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-TAD (DMEQTAD) was superior to MBOTAD in increasing the sensitivity of the resulting derivatives.[19] Based on these results, the derivatization using DMEQTAD was used for the determination of 1α-hydroxyvitamin D_3 [1α(OH)D_3], a synthetic prodrug of 1,25$(OH)_2D_3$, in human plasma.[20] The derivatization condition was very mild; the mixture of 1α(OH)D_3 and DMEQTAD in ethyl acetate was kept at room temperature for 1 h.

Radioreceptor assay and immunoassay are not applicable for the 1α(OH)D_3 measurement, because 1α(OH)D_3 does not have a competent binding affinity to the vitamin D receptor and a specific anti-1α(OH)D_3 antibody with a satisfactory titer is hardly available. For this reason, although 1α(OH)D_3 has been clinically used for the treatment of bone diseases for over 20 years, its pharmacokinetics in humans has been poorly understood.[21] By combination with DMEQTAD-derivatization and acetylation, the LOD [signal to noise ratio (S/N)$=$3] of the derivative was improved to 6.3 fmol [equivalent to 2.5 pg of 1α(OH)D_3] and the LOQ was 25 pg/ml for a 1.0-ml plasma aliquot. The plasma 1α(OH)D_3 was extracted with acetonitrile, purified with disposable cartridges, deriva-

tized and subjected to LC/MS. The method employed the SRM mode, in which the residual $[M+H]^+$ was used as the monitoring ion after the collision of $[M+H]^+$ with 15% of relative energy. This method enabled the determination of the plasma $1\alpha(OH)D_3$ concentrations of a healthy volunteer orally administered 4 μg of $1\alpha(OH)D_3$ (Alfarol® capsule). Thus, with this new LC/MS method, the pharmacokinetic study of $1\alpha(OH)D_3$ in humans became possible.

The DMEQTAD-derivatization was also applied to the characterization of the urinary metabolites of vitamin D_3 in humans under physiological conditions.[22] The urine specimens obtained from healthy volunteers were treated with β-glucuronidase, purified with SPE cartridges, derivatized with DMEQTAD and subjected LC/MS/MS. The derivatization enabled the detection of the metabolites in the picogram range. This study demonstrated that the hydroxylation at the C-23 position is the important side-chain modification of 25-hydroxyvitamin D_3 [$25(OH)D_3$], the circulating form of vitamin D_3, to excrete the excess vitamin D_3 in humans.

6. MS Tag for Electron Capture APCI-MS

In order to make the MS analysis of a trace analyte in biological samples successful, it is very important not only to enhance the ionization efficiency of the target analyte, but to also reduce the background noise derived from the matrices. Compared to the positive-ion mode, the background noise is relatively lower in the negative-ion mode, which results in the improvement of the S/N, that is, the greater sensitivity. However, the loss of a proton from the steroid molecule hardly occurs during the APCI process, and therefore, the usual negative-APCI-MS is less practical for steroid analyses.

Under APCI conditions, gas-phase electrons are provided by the corona discharge and captured by electron-affinitive compounds (Fig. 5). Durng APCI-MS operating in the negative-ion mode, therefore, an ultrahigh sensitivity can be obtained by tagging steroids with electron-affinitive (electron-capturing) moieties. The first remarkable instance for the electron-capturing derivatization for APCI-MS operating in the negative-ion mode was reported by Singh *et al.*[23] They described the LC/APCI-MS analysis of the pentafluorobenzyl (PFB) derivatives of estrogens, where the derivatives generated negative ions ($[M-PFB]^-$) through the loss of the PFB group from the molecular anions, $[M]^-$ (dissociative electron capture), but not the deprotonated ions, $[M-H]^-$. Consequently, they termed this technique electron capture APCI (ECAPCI), which provided an increase in the sensitivity of 2 orders of magnitude, when compared with conventional APCI methodology.

Based on this report, the author examined the electron-capturing ability of the nitrobenzene (NB) moiety and found that it was superior to the PFB group in the sensitivity in ECAPCI.[24] The author then applied 4-(4-nitrophenyl)-TAD (NPTAD) to the LC/ECAPCI-MS analysis of serum $25(OH)D_3$, an index of vitamin D deficiency (Fig. 4).[25] NPTAD, as well as other Cookson-type reagents, quantitatively reacted with $25(OH)D_3$ at room temperature within 1 h. The LOD in this method was 10 fmol [equivalent to 4 pg of intact $25(OH)D_3$], which was 30-times that obtained without derivatization. Furthermore, the author demonstrated that the derivatization of estrogens with 4-nitrobenzenesulfonyl

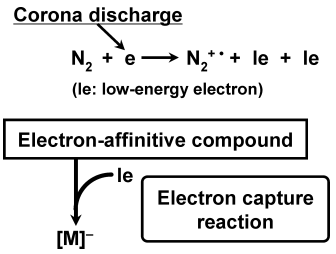

Fig. 5. Principal of ECAPCI

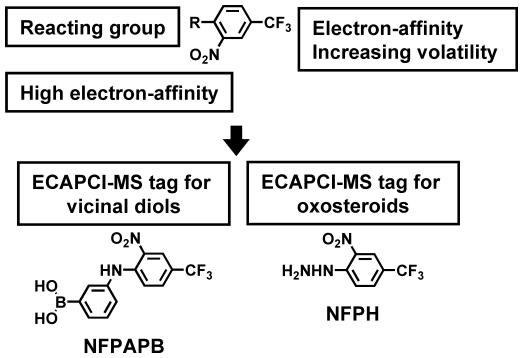

Fig. 6. Newly Developed ECAPCI-MS Tags with NFP Backbone

chloride (in acetone–1 M Na_2CO_3 at 60 °C for 30 min) followed by LC/ECAPCI-MS allowed the reproducible and accurate quantification of the serum and urine estrone and estradiol of a pregnant woman, which is useful for the diagnosis of the fetoplacental function, with small amounts (10 μl) of sample and a simple pretreatment procedure (only deproteinization).[26] The author is now planning the application of this method to the diagnosis and follow-up of the medical treatment of diseases relevant to estrogens, such as breast cancer.

As already mentioned, APCI, including ECAPCI, is a gas-phase ionization process. Therefore, when a less polar structure, which generally has a greater volatility, is used as the backbone of an ECAPCI-MS tag, a higher sensitivity is obtained in the analysis of the resulting derivatives. The NB-based ECAPCI-MS tag with the additional trifluoromethyl group, which shows both the electron-capturing and volatile properties, provided a higher sensitivity than that having only nitro group(s), and the author found that the 2-nitro-4-trifluoromethylphenyl (NFP) structure is the most suitable one for the ECAPCI-MS tag backbone (Fig. 6).[24] The author then designed and synthesized the boronic acid derivative having the NFP structure [3-(2-nitro-4-trifluoromethylphenyl)-aminophenyl]dihydroxyborane (NFPAPB)] for the analysis of vicinal diols.[24] Using this reagent, a highly sensitive method for the determination of 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$], one of the major metabolites of vitamin D_3 expected as a new anti-osteoporosis agent, in human plasma was developed. $24,25(OH)_2D_3$ was derivatized with NFPAPB in pyridine at 50 °C for 1 h. The resulting boronate gave only a very intense $[M]^-$ ion in the negative-ion mode (Fig. 7) and an injection of about 1 fmol of the derivative [equivalent to about 0.4 pg of $24,25(OH)_2D_3$] was readily detected, which showed an over 200-fold higher sensitivity compared to the underivatized form. Only a 50-μl plasma was required for

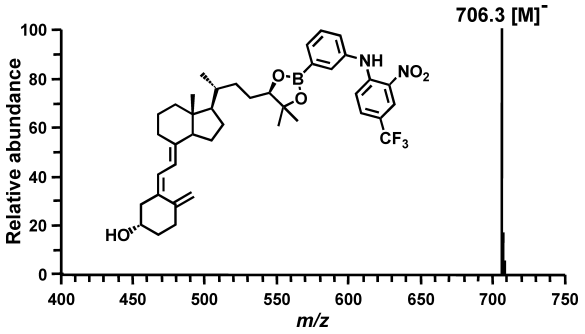

Fig. 7. ECAPCI Mass Spectrum of NFPAPB Derivative of $24,25(OH)_2D_3$

this assay, which was the smallest volume used in hitherto developed methods for the $24,25(OH)_2D_3$ assays, including immunoassays.

NSs, which are synthesized *de novo* in the central nervous system (CNS), affect the neurotransmission through action at the membrane ion-gated and other neurotransmitter receptors.[1] Although the physiological and neurobiological roles of NSs are not yet fully elucidated, recent studies have demonstrated that pregnane-type NSs play an important role in the homeostatic mechanisms that counteract the inhibitory effect of stress on the γ-aminobutyric acid type A ($GABA_A$) receptor function (Fig. 8).[27] Therefore, the down-regulation of the NS synthesizing activity may be involved in the stress-elicited disorders, such as depression.[28] In addition, there is evidence that the anti-depressant activity of selective serotonin reuptake inhibitors (SSRIs), such as fluoxetin, occur through the promotion of the brain NS synthesis.[29] With this background information, a method for the determination of NSs in the animal brains under various conditions, especially stressful conditions, can contribute to the elucidation of their physiological roles and the development of new antipsychotic agent targeting neurosteroidogenesis.

The author developed 2-nitro-4-trifluoromethylphenylhydrazine (NFPH) as the ECAPCI-MS tag for oxosteroids[24] and used it for the analysis of the pregnane-type NSs in rat brains.[30,31] The mixture of NSs, NFPH and trichloroacetic acid (catalyst) was subjected to an ultrasonic treatment at ambient temperature for 1.5 h to convert NSs to the ECAPCI-active derivatives. The NFPH derivatives of PREG and PROG provided intense $[M]^-$ ions in the negative-ion mode, and their LODs were 19 and 3.2 fmol (equivalent to 6 pg of PREG and 1 pg of PROG), which were 20- and 30-times superior to those obtained without derivatization, respectively. This derivatization–LC/ECAPCI-MS method had a great power in reducing the background noise. As shown in Fig. 9, when the underivatized sample was subjected to LC/positive-APCI-MS, many large peaks derived from the endogenous brain components interfered with the analysis of the brain PREG. On the other hand, when the brain PREG was analyzed as its NFPH derivative by LC/ECAPCI-MS, little background noise was observed in the chromatogram. Furthermore, the method based on the derivatization and ECAPCI-MS make it possible to determine the brain NSs with a small sample volume (in this case, 10 mg), compared to the usual LC/positive-APCI-MS method (50 mg).

Changes in the NS levels in the rat brains due to immobilization stress, a representative physical stress, were analyzed

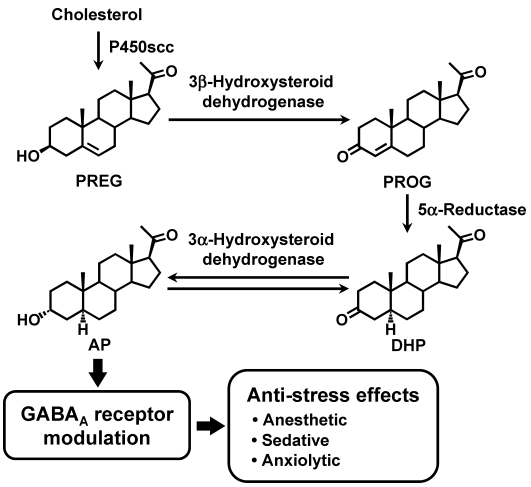

Fig. 8. Biosynthesis and Action of Pregnane-Type NS

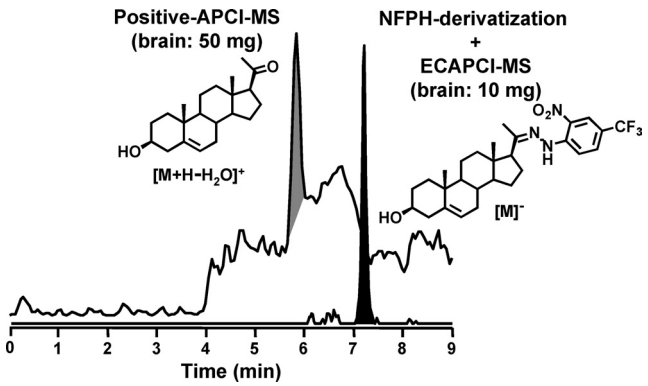

Fig. 9. Usefulness of the NFPH-Derivatization and ECAPCI-MS during Analysis of Brain PREG

Table 2. Changes in NS Levels by Immobilization Stress

NS	Brain level (ng/g tissue)[a]	
	Untreated	Stressed
PREG	7.4±1.8	60.7±12.8
PROG	<LOQ[b]	13.1±7.2
DHP	Not detected	3.1±1.4
AP	Not detected	1.9±1.0

a) Mean±standard deviation (n=5). *b*) Not detected or <LOQ (0.5 ng/g tissue).

using the present method.[31] PREG was detected as its NFPH derivative in both the untreated and stressed rats and the brain levels in the stressed group were much higher than those in the untreated group (Table 2). NSs, including PREG, have been reported to exist as not only the free forms, but also as various conjugates, such as fatty acid esters.[32] Shimada and Mukai have reported that a large amount of PREG stearate, a representative fatty acid ester of PREG, is present in the rat brains and its level significantly decreased by the immobilization stress.[33] Based on these data, the fatty acid esters of PREG are inferred to be storage forms and rapidly converted to PREG depending on the situation, such as stressful conditions.

The brain level of PROG was changed more dramatically than that of PREG by the immobilization stress. That is, the

PROG levels of the untreated group were less than the limit of quantitation (LOQ; 0.5 ng/g tissue) even if the 100 mg of brain tissue was used, whereas those of the stressed group were within the range of 5—23 ng/g tissue, which were easily measured with only 25 mg of the brain tissue (Table 2). Although the 5α-reduced metabolites, 5α-dihydroprogesterone (DHP) and AP, were not detected in the brains of the untreated group, they were produced in the brain of the stressed group.

It is known that various types of stress lowers the GABAergic transmission, induces depolarization of the cell and neuronal overexcitation, and also causes hypomnesia and depression.[27] Therefore, the living body accelerates the brain synthesis of AP, which potentiate the GABA-induced chloride influx through the ion channel by prolonging the duration of the opening and increasing the opening frequency and counteracts the neuronal overexcitation elicited by the stress. The increase in the brain AP level observed here is certainly this described defensive response against acute stress. With the acceleration of the AP synthesis, the brain levels of PREG, PROG and DHP, the precursors of AP, also increase.

7. Conclusion

Compared to GC/MS, the derivatization procedure is not essential for LC/MS, which is one of the advantages of this technique. However, one of the limitations for LC/MS is the poor ionization behavior of some compounds of biological interest, such as steroids, leading to a low sensitivity. The development of practical derivatization methods for steroids is necessary to overcome this problem and widen the applicability of LC/MS. The detection-oriented derivatization in LC/MS will become an important field of analytical chemistry as demand for ultrasensitive analyses increases. The derivatization step surely decreases the analysis throughput, but it can provide a massive amount of new biological information.

In summary, the detection-oriented derivatization procedures suitable for the respective ionization methods are as follows. For ESI-MS, the introduction of permanently charged moieties is effective. The introduction of proton-affinitive moieties enhances the analyte signals in the positive APCI-MS. ECAPCI-MS based on the introduction of an electron-affinitive group will be applied to various steroids due to its high sensitivity and easy performance using a commercial APCI interface in the negative-ion mode. References on the sensitivity enhancement in LC/MS are given at the end of this paper.[34—36] These articles describe the various chemical approaches (electrochemical conversion, mobile phase additives as well as derivatization) to improve the sensitivity in both the ESI and APCI detection modes.

Acknowledgments First of all, the author would like to express his sincere respect and gratitude to Professor Kazutake Shimada, Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, for his kind and pertinent advice and encouragement during these studies. The author would like to express his appreciation to Professor Norihiro Kobayashi (Kobe Pharmaceutical University), Dr. Kuniko Mitamura (Kinki University) and Mr. Tadashi Nishio for their kind support, advice and encouragement throughout the studies. The author also expresses his appreciation to emeritus Professor Toshio Nambara (Tohoku University) for his kind encouragement. Thanks are also due to Professor Mikio Namiki (Kanazawa University), Professor Hideo Honjo (Kyoto Prefectural University of Medicine), Professor Susumi Hatakeyama (Nagasaki University), Dr. Eitetsu Koh (Kanazawa University), Dr. Seijiro Honma (Teikoku Hormone Medical Research Center) and Dr. Noboru Kubodera (Chugai Pharmaceutocal Co., Ltd.) for their valuable advice and supplying materials and samples. The studies were made possible by the hardwork of the coworkers whose names are shown in the papers, and valuable supports and discussions with the lab members and collaborators. Financial support from Japan Society for the Promotion of Science (JSPS), Kanazawa University (Special Grants) and Takeda Science Foundation is greatly appreciated.

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