

Focusing Review

Development of Analyses of Biological Steroids Using Chromatography
—Special Reference to Vitamin D Compounds and Neurosteroids—

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Abstract

Steroids comprise a large group of natural substances that must frequently be monitored in various biological materials. Due to the metabolic versatility of steroid molecules, extremely complex mixtures are often encountered, necessitating the use of a chromatographic procedure prior to measurement. In this article we present our work, that is, the development of analyses of biological steroids (especially vitamin D compounds and neurosteroids) using gas chromatography/mass spectrometry, high-performance liquid chromatography (including inclusion chromatography using cyclodextrin) and liquid chromatography/mass spectrometry.

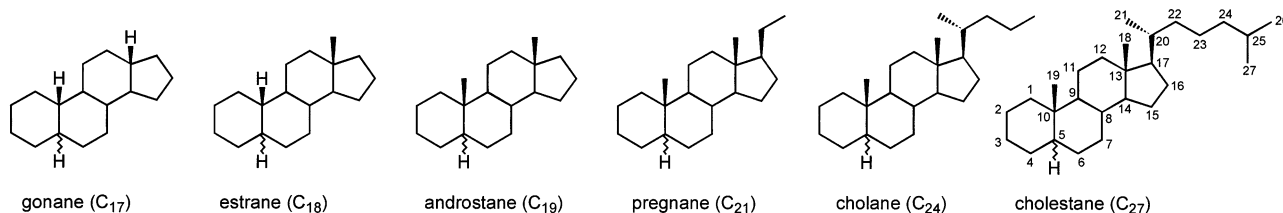
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1. Introduction

The basic steroid molecular skeleton consists of four rings of carbon atoms, perhydro-1, 2-cyclopentenophenanthrene. Almost all natural steroids possess either one or, more usually two, methyl groups at 'angular' positions where two rings meet. The steroids with which we shall be mainly concerned are of six skeletal types according to the number of C-atoms, gonane (C₁₇), estrane (C₁₈), androstane (C₁₉), pregnane (C₂₁), cholane (C₂₄) and cholestane (C₂₇) as shown in Fig. 1. Almost all these compounds are natural hormones or precursors, and steroid hormones can be divided into androgens, corticoids and estrogens according to their functions. The other steroids such as bile acids (cholane), vitamin D compounds

(9, 10-secosteroids) and cardiac steroids having an α , β -unsaturated ring at the 17 β -position also have biologically important activities [1, 2].

Steroids comprise a large group of natural substances that must frequently be monitored in various biological materials. Additionally, numerous synthetic steroids have been used as therapeutic agents. Due to the metabolic versatility of steroid molecules, extremely complex mixtures are often encountered, necessitating the use of a chromatographic procedure prior to measurement [2]. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are commonly used separation and determination procedures, but the former is applicable only to volatile steroids and

**Figure 1.** Structures of steroids

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not to non-volatile ones such as conjugates. GC/mass spectrometry (MS) is also widely used for the determination of steroids, but liquid chromatography (LC)/MS is recently considered to be the most promising analytical method for the determination of steroids including conjugated ones due to its sensitivity, specificity and versatility [3, 4].

In this article we present our work, that is, the development of analyses of biological steroids (especially vitamin D compounds and neurosteroids) using GC/MS, HPLC (including inclusion HPLC using cyclodextrin as a mobile phase additive) and LC/MSⁿ with ion-trap MS.

2. Analysis of vitamin D compounds

The so-called "vitamin D (D)" actually consists of two different compounds, D₃ and D₂, which differ in their side-chain structure at the 17 β-position of the secosteroid. Both D₃ and D₂ are metabolized in the same way in the human body and show similar biological activity. The values of D₂ and its metabolites in biological fluids are usually less than one-tenth of those of D₃ and its metabolites. D is hydroxylated in the liver to 25-hydroxyvitamin D [25(OH)D], which is further metabolized in the kidney either to 1α, 25-dihydroxyvitamin D [1, 25(OH)₂D] or to other compounds, such as (24*R*)-24, 25-dihydroxyvitamin D [24, 25(OH)₂D]. 25(OH)D is the major circulating metabolite, and 1, 25(OH)₂D is

the most potent metabolite for the stimulation of intestinal calcium transport, the mobilization of calcium from the bone and for the prevention of rickets. The induction of cell differentiation and immunoregulation by 1, 25(OH)₂D was also reported, and much interest has been focused on this metabolite and its analogues as potential antileukemia agents (Fig. 2) [5].

2.1. Metabolism of 24, 25-dihydroxyvitamin D₃

In the D-supplemented state, 25(OH)D₃ is metabolized to various side-chain dihydroxylated metabolites, such as 24, 25(OH)₂D₃ and (23*S*)-23, 25-dihydroxyvitamin D₃. The physiological roles of these dihydroxylated metabolites still remain poorly understood. For example, 24, 25(OH)₂D₃ was reported to cause a marked increase in bone volume and mechanical strength in animals without hypercalcemia at pharmacological doses and is expected to be an anti-osteoporosis medicine; however, it is argued to be a catabolic metabolite to be excreted. A study of the metabolism of D is expected to be helpful in understanding the physiological role of the metabolites or in developing a new medicine.

24, 25(OH)₂D₃ is reported to be further oxidized on its side-chain at the 23- or 24-position to give metabolites such as 25-hydroxy-24-oxovitamin D₃ and (23*S*)-23, 25-dihydroxy-24-oxovitamin D₃ [23, 25(OH)₂-24-oxo-D₃]. Thus, the phase I reactions in the D metabolism, particularly the oxidation of the side-chain has been investigated thoroughly, but a little is known regarding the conjugates of D metabolites, such as glucuronide (G) or sulfate (S) [6,7].

2.2. New metabolite—3-Epimer—

We clarified the nature of the conjugated D metabolites in rat bile after the administration of 24, 25(OH)₂D₃; new metabolites (peaks A, B, C and monoglucuronides of 24, 25(OH)₂D₃ in Fig. 3) were isolated [8]. The Peaks A [23, 25(OH)₂-24-oxo-D₃ 23 G], B [3-epi-24, 25-dihydroxyvitamin D₃ [3-epi-24, 25(OH)₂D₃] 24 G] and C [24, 25(OH)₂D₃ 3 S] were identified using HPLC with photodiode array UV detection, LC/MSⁿ combined with derivatization using a Cookson type reagent [4-phenyl-1, 2, 4-triazoline-3, 5-dione (PTAD)] (Fig. 4) and GC/MS. 24, 25(OH)₂D₃ 3- and 24-G were identified by comparison with synthetic samples [9] in their chromatographic behavior. LC/MSⁿ combined with derivatization using a Cookson type reagent (PTAD) was very effective to identify Peak B as 3-epi-24, 25(OH)₂D₃ 24 G. That is, LC/MSⁿ analysis of intact 3-epi-24, 25(OH)₂D₃ 24 G gave inconclusive information concerning its conjugated position, but the PTAD derivative gave the fragment ion concerning the position in MS³ spectrum. Inclusion HPLC using γ-cyclodextrin as a mobile phase additive was also effective for the separation of 3-epi-24, 25(OH)₂D₃, which was synthesized as an authentic sample [8], and its epimer [24,

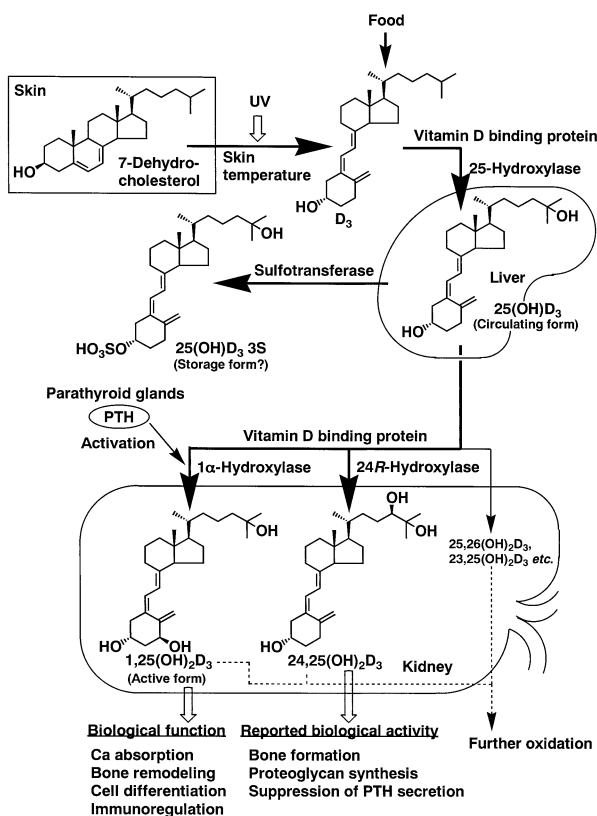


Figure 2. Biosynthetic and metabolic pathway of D₃

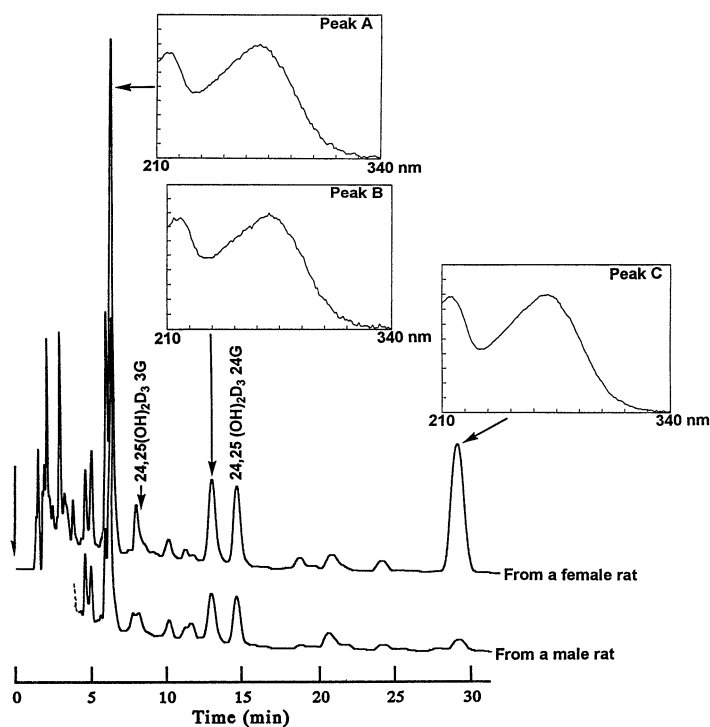


Figure 3. Representative HPLC chromatograms and UV spectra of conjugated metabolites in bile of rats administered 24, 25(OH)₂D₃

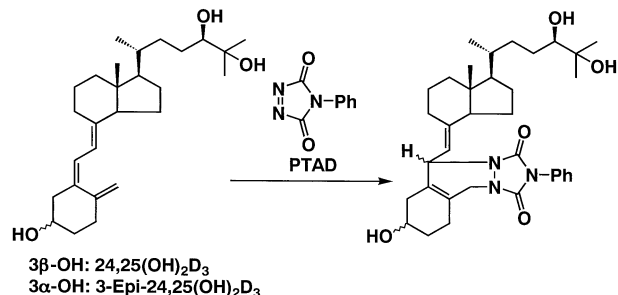


Figure 4. PTAD adducts of 24, 25(OH)₂D₃ and 3-epi-24, 25(OH)₂D₃

25(OH)₂D₃] [10].

In this manner we found a new metabolic pathway in which the 3-hydroxy group of 24, 25(OH)₂D₃ was epimerized, which acts as one of the important pathways in D metabolism (Fig. 5).

2. 3. Determination of 1 α-hydroxyvitamin D₃

1 α-Hydroxyvitamin D₃ [1 α(OH)D₃] is now well-known as a synthetic pro-drug (ALFAROL[®]) of 1, 25(OH)₂D₃, and has been clinically used for the treatment of rickets, hypovitaminosis, hypocalcemia, chronic renal fail-

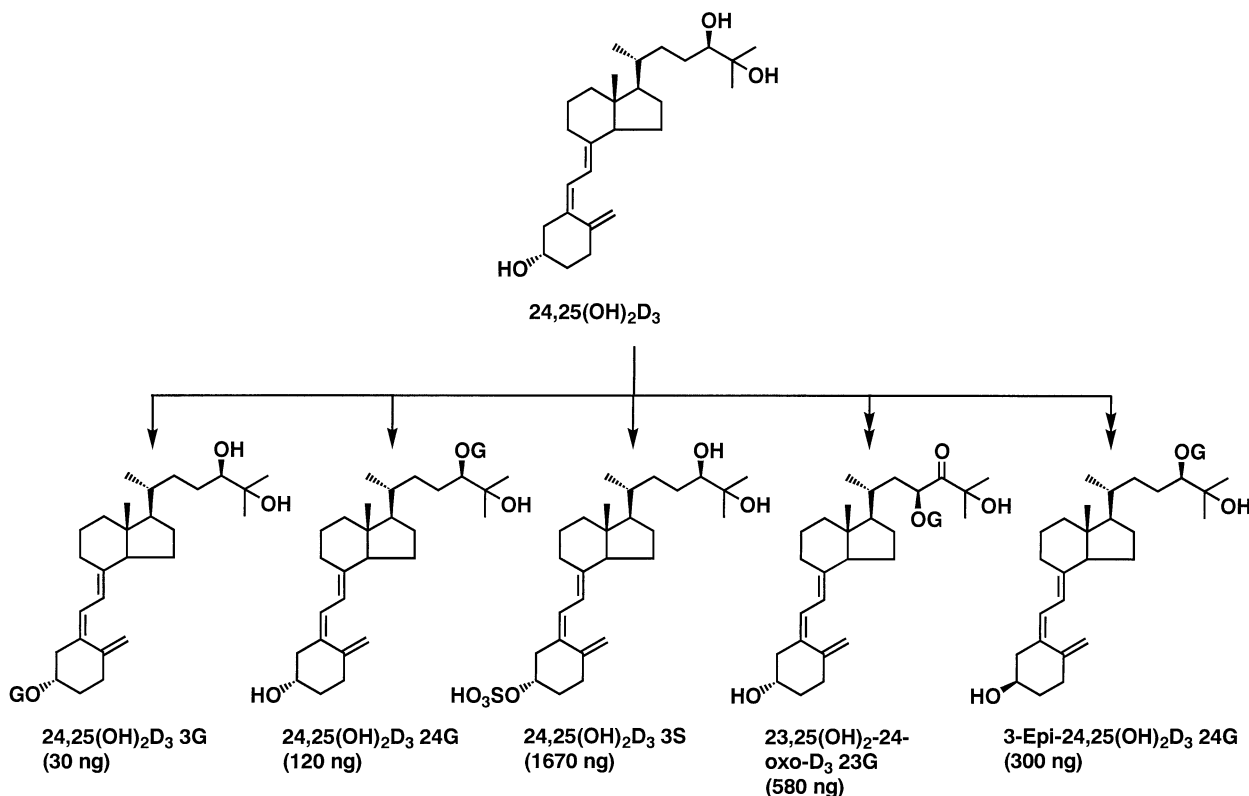


Figure 5. Metabolic pathway of 24, 25(OH)₂D₃.

Values in parentheses are approximate yields of each compound from 1 ml of bile.

ure and osteoporosis [11]. Although there have been a few papers which describe the change in the plasma/serum concentration of 1, 25(OH)₂D₃ after the administration of 1 α(OH)D₃ (oral dose: several μg per person), the plasma/serum concentration of 1 α(OH)D₃ has not been reported. Recently, the pharmacokinetics of 1 α(OH)D₃ in the rat were investigated using a tritium-labeled compound with high specific radioactivity [12]. This method is highly sensitive, but it requires special equipment due to the use of a radioisotope (RI). Therefore, a practical non-RI method for the determination of 1 α(OH)D₃ in biological fluids is strongly required, though developing the method is one of the most challenging subjects in the field of D analysis.

A stable isotope dilution LC/MS² method for the determination of plasma 1 α(OH)D₃ has been developed [11]. The method employed derivatization, the reaction with a Cookson type reagent, 4-[2-(6, 7-dimethoxy-4-methyl-3-oxo-3, 4-dihydroquinoxalyl)ethyl]-1, 2, 4-triazoline-3, 5-dione (DMEQTAD) and acetylation (Fig. 6), which significantly improved the ionization efficiency of 1 α(OH)D₃ with a detection limit of 6.3 fmol per injection in atmospheric pressure chemical ionization (APCI)-MS. The plasma 1 α(OH)D₃ was extracted with acetonitrile, purified with disposable cartridges, derivatized and subjected to LC/MS² analysis. The intra- and inter-assay coefficients of variation (n=5) were below 10.6 and 4.7%, respectively, and the analytical recovery of 1 α(OH)D₃ was quantitative. The limit of quantification, which was determined with its detection limit (signal/noise; S/N=3) and calibration curve, was 25 pg/ml for a 1.0 ml plasma aliquot. The application of the developed method to a plasma sample of a volunteer given 1 α(OH)D₃ (4 μg) orally was also done. Although the pharmacokinetics of 1 α(OH)D₃ in humans have been indirectly evaluated by the change in the plasma/serum 1, 25(OH)₂D₃ levels as described above, the direct pharmacokinetic study of 1 α(OH)D₃ in humans can be carried out using this new LC/MS² method

3. Analysis of neurosteroids

Since the discovery of dehydroepiandrosterone in rat brains, several 17- and 20-oxosteroids, called neurosteroids, have been elucidated in mammalian brains [13]. The term "neurosteroids" ap-

plies to those steroids that are both synthesized in the nervous system, either *de novo* from cholesterol or from steroid hormone precursors, and that accumulate in the nervous system to levels that are at least in part independent of the steroidogenic gland secretion rates. The neurosteroids that exist in rat brains are reported as the free form, sulfates, lipoidal esters and sulfolipids [14]. Much interest is now focused on the physiological significance of these steroids and the term "neuroactive neurosteroids" is also used for these steroids [13-15].

3. 1. Pregnenolone 3-sulfate in rat brains

Pregnenolone 3-sulfate (PS) is known as one of the neurosteroids (Fig. 7); it acts as a positive and negative modulator of the NMDA (*N*-methyl-D-aspartate) and GABA_A (γ-aminobutyric acid) receptor, respectively [16]. An intensive investigation of its biosynthetic pathway and biological properties has been done. Although the quantitative determination of PS in rat brains has been already done with GC/MS or radioimmunoassay, these were based on indirect methods which determined the liberated genin after the solvolysis [14, 17]. Therefore, we developed a quantitative determination method of PS in rat brains without deconjugation using derivatization followed by LC/electrospray ionization (ESI)-MSⁿ [18].

3. 2. LC/MS of pregnenolone 3-sulfate and its derivatives

The ESI-MS of PS showed the dehydrated ion [M-H]⁻ as the base peak; however, ESI-MS and -MS² did not give satisfactory sensitivity to establish a determination method of PS in rat brains. It is experimentally known that compounds having proton-affinitive atoms, such as oxygen and nitrogen, are excellent substrates for detection with APCI-MS operating in the positive-ion mode, and we previously reported that the derivatization of oxosteroids into a methyloxime gave the satisfactory results in LC/ESI-MS analysis of pregnenolone in rat brains [19]. These results prompted us to use the derivatization method for the quantitative determination of PS using LC/ESI-MS. The commercially available *O*-methylhydroxylamine, 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2, 1, 3-benzoxadiazole (DBD-H) and *O*-pentafluoroben-

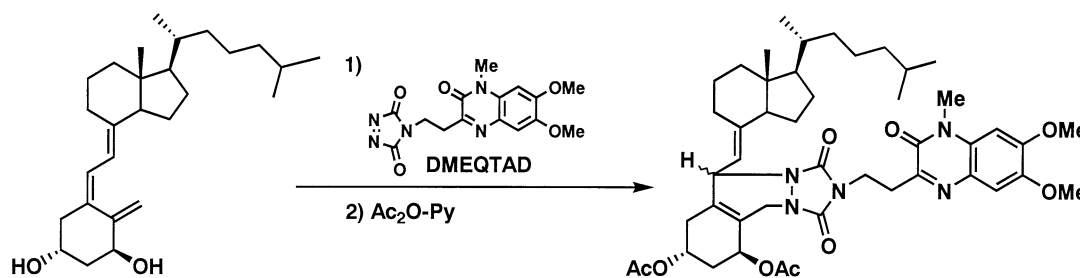


Figure 6. Structures of ALFAROL[®] and its derivatives

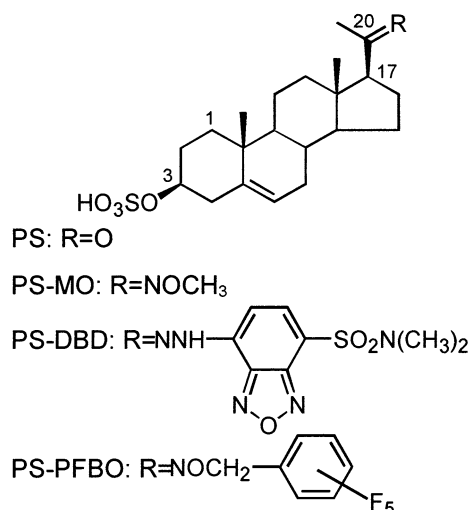


Figure 7. Structures of PS and its derivatives

Table 1. Comparison of relative sensitivity in PS and its derivatives

Compound	MW	Base peak	Relative sensitivity ^a
PS	396	395 [M-H] ⁻	1
PS-MO	425	424 [M-H] ⁻	9
PS-DBD	635	634 [M-H] ⁻	8
PS-PFBO	591	590 [M-H] ⁻	11

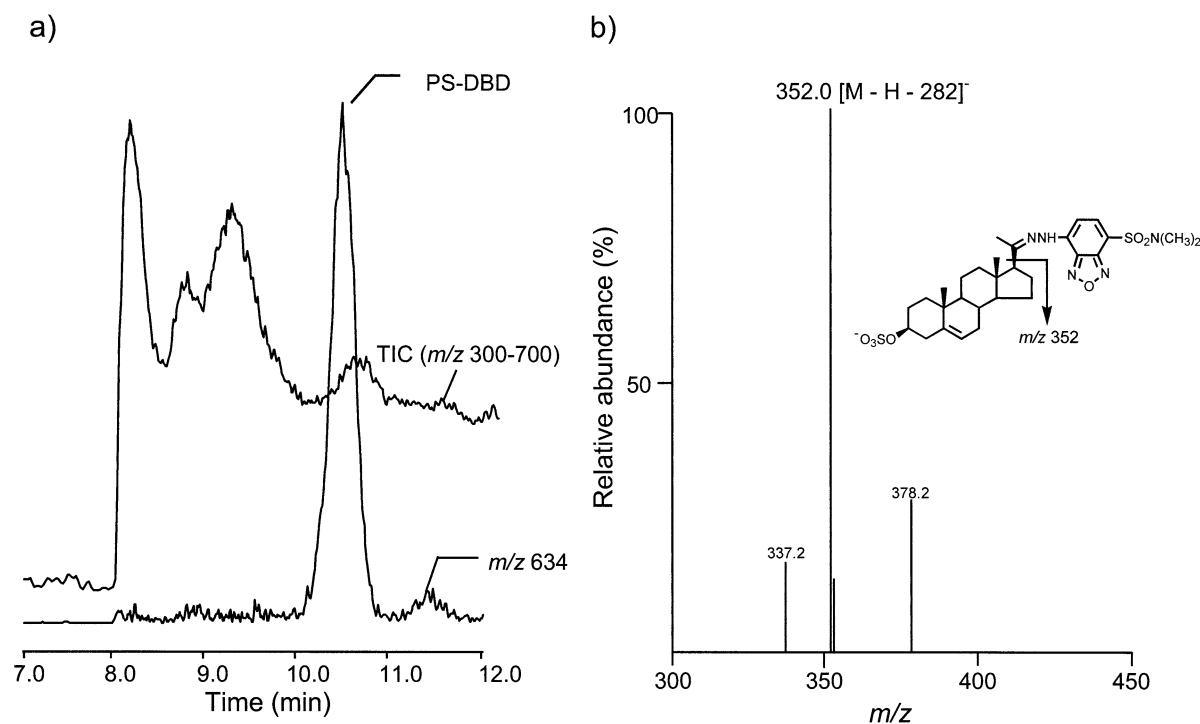
^a The relative sensitivity of each derivative against PS was calculated using the peak area of the base peak ion measured by SIM.

zylhydroxylamine were selected as the derivatization reagents which reacted with the carbonyl group of PS to give PS-methyloxime (MO), -DBD and -pentafluorobenzylloxime (PFBO), respectively (Fig. 7).

The obtained ions and relative sensitivity between PS and its derivatives using the peak area of the base ion measured by selected ion monitoring (SIM) are summarized in Table 1. The data showed that the PS derivatives exhibited the [M-H]⁻ ion as the base ion, and that the detection responses of the derivatives were increased by 8 to 11 fold over that of PS. Of these reagents, DBD-H was the most useful in its reactivity with PS and the chromatographic behavior of the resulting derivative; therefore, this reagent was used for the following experiments.

3.3. Quantitative determination of pregnenolone 3-sulfate in rat brains

The brains of adult Wistar strain rats were homogenized and extracted with ethanol, and the extract was subsequently purified by solid-phase extraction and ion-exchange chromatography. The fraction containing PS was derivatized with DBD-H followed by purification with solid-phase extraction; then the obtained residue was applied to an LC/ESI-MS in the total ion monitoring mode (Fig. 8 a; TIC). PS was identified by comparison with authentic PS-DBD based on its mass chromatographic behavior. LC/ESI-MS² (precursor ion, *m/z* 634 [M-H]⁻, relative collision energy,

Figure 8. LC/ESI-MSⁿ data of PS in rat brains as DBD derivative

a) Mass chromatogram, b) product ion mass spectrum

30%) also gave the characteristic product ion m/z 352 ($[M-H-282]^+$), which was produced by dissociation of the $C_{17}-C_{20}$ bond (Fig. 8 b). However, the obtained peak intensity was not high enough to determine PS in smaller amounts of brain, so the collision energy was reduced to 10% and the residual $[M-H]^+$ ion (m/z 634) was selected as the monitoring ion (Fig. 8 a). In this system, the desired peak was detected without interfering peaks; the detection limit of PS was 80 pg/injection ($S/N=5$). We calculated the concentration of PS in the brains of male Wistar strain rats using the above method combined with the isotope dilution and standard addition methods. $[7, 21, 21, 21-^2H_4]$ PS was used as the internal standard (IS) and the derivatization with DBD-H protected the deuterium-hydrogen exchange reaction of IS during the ionization procedure. The developed method does not require solvolysis and proved to be satisfactory in its accuracy and precision. The obtained concentrations were 0.53 ± 0.28 ng/g tissue (mean \pm standard deviation, $n=10$) [18], which were much lower than those reported in previous studies (*ca.* 20 ng/g tissue) [14,17]. These data prompted us to develop the enzyme-linked immunosorbent assay for examining whether PS is a veritable neurosteroids; the obtained results are lower than those obtained from the above LC/MS [20].

4. Conclusion

In this article we present the development of analyses of D compounds and neurosteroids in biological materials using GC/MS, HPLC and LC/MSⁿ. Inclusion chromatography using cyclodextrin as a mobile phase additive is effective for the separation of stereoisomers. A suitable derivatization method provides a more sensitive and selective analysis of D compounds and neurosteroids using LC/MSⁿ. The derivatization also gives much more structural information and protects the deuterium-hydrogen exchange reaction, such as $[^2H_4]$ PS (IS) with DBD-H, in LC/MSⁿ analysis [21].

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