Focussing Review

# Derivatization in Liquid Chromatography/Mass Spectrometric Analysis of Neurosteroids

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**Abstract**

Liquid chromatography/mass spectrometry (LC/MS) is now considered to be the most promising analytical method for the determination of biological substances, especially nonvolatile or highly polar substances. However, some compounds do not show enough sensitivity in LC/MS and soft-ionization methods commonly used in LC/MS, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), sometimes do not give satisfactory structural information. This report presents an overview of the derivatization methods in the LC/MS analysis of neurosteroids or neuroactive neurosteroids, which are synthesized and accumulated in the nervous system. The derivatization of pregnenolone 3-sulfate, one of these steroids, with 4-(*N*,*N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole gave a satisfactory sensitivity during the quantitative analysis using LC/ESI-MS. The obtained results are much lower than those previously obtained using gas chromatography/MS or radioimmunoassay. On the other hand, the derivatization to acetate was useful for the treatment of labile catechol estrogens in rat brains and gave enough structural information in LC/APCI-MS, which confirmed the existence of catechol estrogens in mammalian brains.

*Keywords*: liquid chromatography/mass spectrometry, derivatization, neurosteroid, pregnenolone 3-sulfate, 4-(*N*,*N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole, catechol estrogen, acetate

## 1. Introduction

Gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS), representatives of the hyphenated techniques, are some of the most reliable analytical methods, which are the synergistic combination of two powerful analytic techniques; the chromatograph separates the components of a mixture in time, and the mass spectrometer provides information that aids in the structural identification of each component. Since the introducing of a good and practical interface combined with an ionization procedure, LC/MS has developed into a mature technique, which can be routinely applied in a large number of application areas, *e.g.*, pharmaceutical, industrial and environmental chemistry. The commonly used ionization methods in LC/MS are the so-called soft-ionization methods, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The former and the latter are suitable for ionic and neutral compounds, respectively. LC/MS is now considered to be a promising analytical method for the determination of biological substances, especially thermally unstable, nonvolatile, highly polar and/or highly molecular weight compounds. However, some compounds do not show enough sensitivity in the LC/MS and soft-ionization methods, such as ESI and APCI, sometimes give fewer fragment ions, therefore, it is unsuitable for structural elucidation. Although the instruments, such as micro LC and tandem MS ($MS^2$), have been introduced, these are not always satisfactory to overcome these problems. On the other approach, it is expected that the derivatization is also effective to improve the sensitivity and obtain more abundant structural information on LC/MS, whose techniques are commonly used and give satisfactory results on GC/MS[1, 2]. In this paper, the advantage of the derivatization method in the LC/MS analysis of neurosteroids is described.

## 2. Neurosteroids

Since the discovery of dehydroepiandrosterone in rat brains,

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several 17- and 20-oxosteroids called neurosteroids, have been elucidated in mammalian brains[3]. The term "neurosteroids" applies 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[4]. Much interest is now focused on the physiological significance of these steroids and the term "neuroactive neurosteroids" is also used for these steroids[1, 3-5].

### 3. Determination of pregnenolone 3-sulfate in rat brains

Pregnenolone 3-sulfate (PS) is known as one of the neurosteroids (Figure 1), and it acts as a positive and negative modulator of the NMDA and $GABA_A$ receptor, respectively[6]. 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 indirect methods which determined the difference in the liberated genin before and after the solvolysis[4, 7]. We previously clarified the existence of PS in rat brains using LC/ESI-MS without solvolysis[8], however, a satisfactory sensitivity for quantitative determination was not obtained. Therefore, we developed a quantitative determination method of PS in rat brains using derivatization followed by LC/ESI-$MS^n$ with ion-trap MS[9].

#### 3.1. LC/MS of PS and its derivatives

The ESI-MS of PS showed the dehydrated ion $[M-H]^-$ as the base peak, however, this MS and ESI-$MS^2$ did not give satisfactory sensitivity to establish the determination method of PS in the rat brains. Although 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, we previously reported that the derivatization of oxosteroids into methyloxime strongly increased their sensitivity in LC/ESI-MS in the negative-ion mode[8]. 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*-pentafluorobenzylhydroxylamine were selected as the derivatization reagents which reacted with the carbonyl group of PS to give PS-methyloxime (MO), -DBD and -pentafluorobenzyloxime (PFBO), respectively (Figure 1).

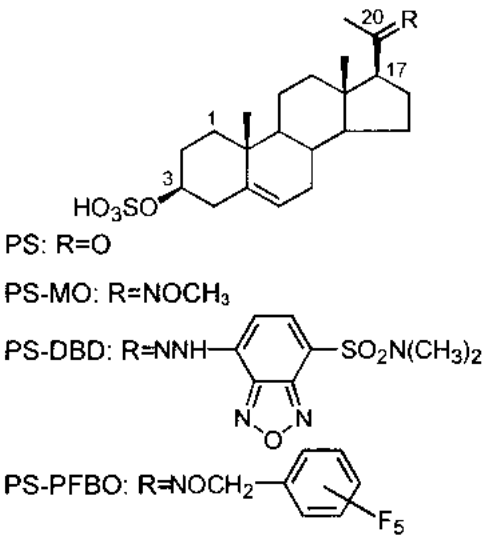



**Figure 1.** Structures of PS and its derivatives[9].

The obtained ions and relative sensitivity between PS and its derivatives using the peak area of the base peak 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 peak, and the detection responses of the derivatives were increased by 8 to 11 fold over that of PS. In 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.

#### 3.2. Quantitative determination of PS 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, and then the obtained residue was applied on an LC/ESI-MS in the total ion monitoring mode. PS was identified by comparison with authentic PS-DBD based on its mass chromatographic behavior as shown in Figure 2a. LC/ESI-$MS^2$ (precursor ion, *m/z* 634 $[M-H]^-$, relative collision energy, 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 (Figure 2b). However, the obtained peak intensity was not high

**Table 1.** Comparison of relative sensitivity in PS and its derivatives[9].

| 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.

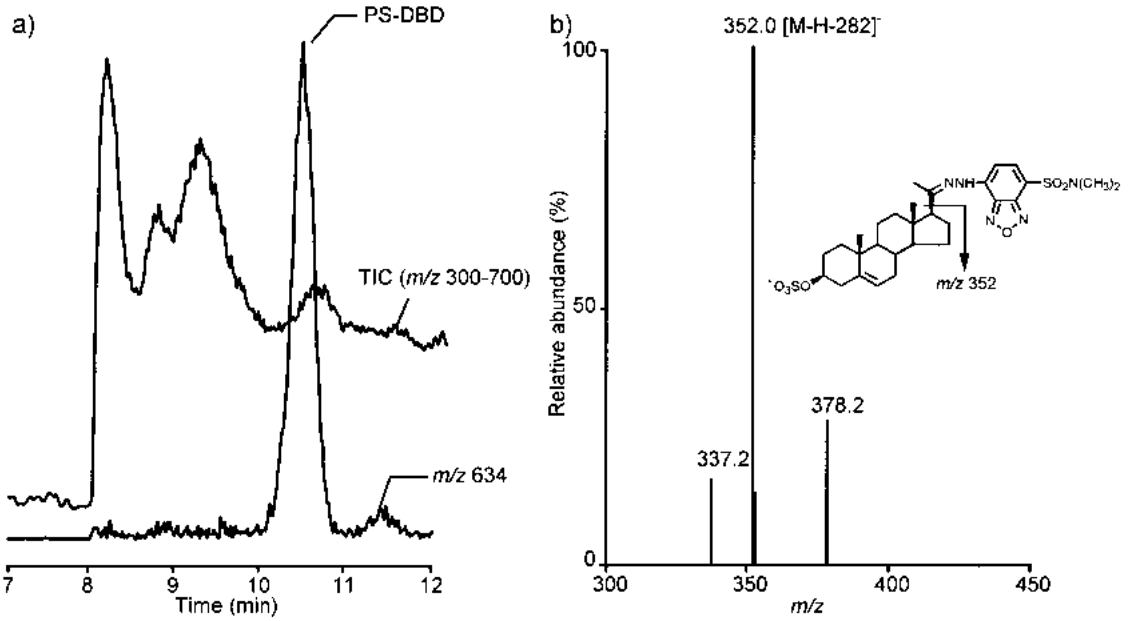
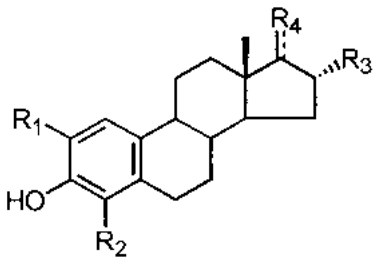



**Figure 2.** LC/ESI-MS$^n$ data of PS in rat brains as DBD derivative. a) Mass chromatogram, b) product ion mass spectrum[9]. Column, YMC-Pack ProC18 (150 x 3.0 mm i.d.); mobile phase, MeOH-4 mM $AcONH_4$ (3:1); flow rate, 0.4 ml/min; b) precursor ion, *m/z* 634 $[M-H]^-$; relative collision energy, 30%.

enough to determine PS in smaller amounts of brain, so the collision energy was reduced to 10% and the residual $[M-H]^-$ ion was selected as the monitoring ion. In this system, the desired peak was detected without interfering peaks; the detection limit of PS was 80 pg/injection (signal/noise=5). We calculated the concentration of PS in the brains of male Wistar strain rats using the above method, the isotope dilution method and standard addition method. $[^2H_4]$-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±standard deviation, n=10), which were much lower than those reported in previous studies (*ca*. 20 ng/g tissue)[4, 7]. Although the reason for this discrepancy is not clear, these results suggest that a more sensitive method is necessary to determine PS in the parts of the brain (< 50 mg tissue).




2OHE$_1$: $R_1$=OH, $R_2$=$R_3$=H, $R_4$=O
4OHE$_1$: $R_1$=$R_3$=H, $R_2$=OH, $R_4$=O
2OHE$_2$: $R_1$=OH, $R_2$=$R_3$=H, $R_4$=β-OH
4OHE$_2$: $R_1$= $R_3$=H, $R_2$=OH, $R_4$=β-OH
2OHE$_3$: $R_1$=$R_3$=OH, $R_2$=H, $R_4$=β-OH
4OHE$_3$: $R_1$=H, $R_2$=$R_3$=OH, $R_4$=β-OH

**Figure 3.** Structures of catechol estrogens.

**4. Identification of catechol estrogens in rat brains**

The usefulness of estrogen-replacement therapy for dementia of the Alzheimer type[10] and the existence of estrogen receptors in the brain have recently been reported[11]. and much interest is focused on the action of estrogens on the central nervous system and on the existence of estrogens in the brain. In a previous paper, we reported the existence of the classical estrogens [estrone ($E_1$), estradiol ($E_2$), estriol ($E_3$)] and the guaiacol estrogens (2-hydroxyestrone 3-methyl ether, 4-hydroxyestrone 3-methyl ether) in rat brains using GC/MS$^n$[12]. This information suggests that the classical estrogens are hydroxylated to the catechol estrogens, the 2- and 4-hydroxylated metabolites, and then *O*-methylated to the guaiacol estrogens in the brain, as well as in other peripheral organs such as the liver.[13, 14]. However, ambiguity still remains regarding the existence of catechol estrogens [2- and 4-hydroxyestrone (2/4-

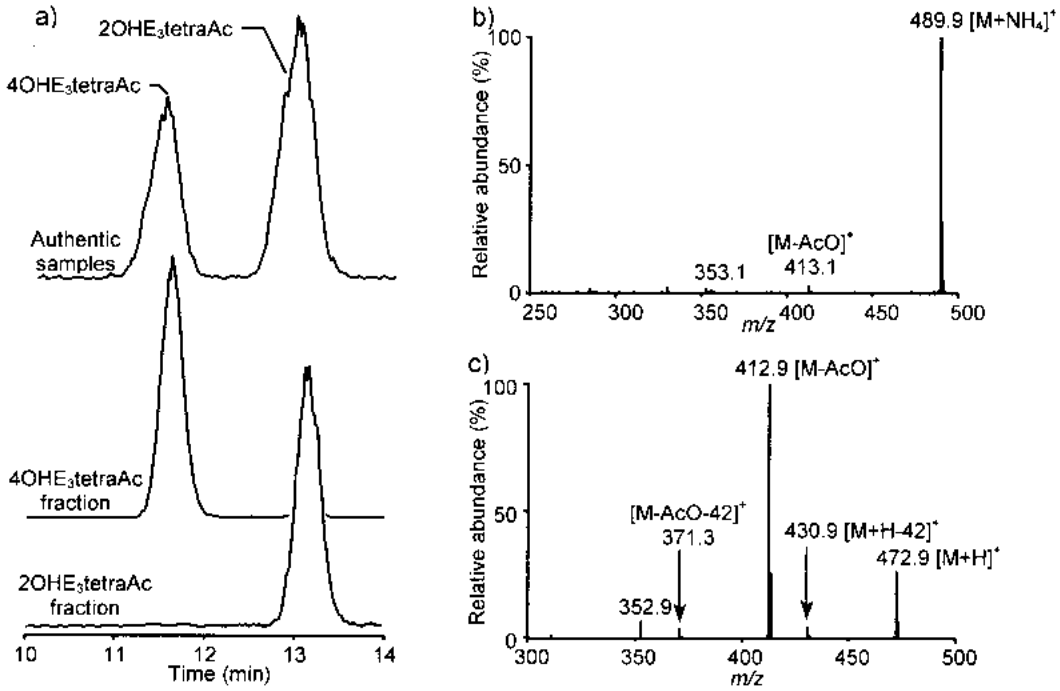



**Figure 4.** LC/APCI-MS$^n$ data of 2/4-OHE$_3$ in rat brains as tetra-acetates. a) Mass chromatograms of 2/4-OHE$_3$tetraAc monitored at *m/z* 490 $[M+NH_4]^+$; b) mass spectrum and c) product ion mass spectrum of 4OHE$_3$tetraAc fraction. Column, J'sphere ODS-H80 (150 x 4.6 mm i.d.); mobile phase, MeOH-$H_2O$ (2:1) containing 0.1% $HCO_2NH_4$; a, b) total ion monitoring; c) precursor ion, *m/z* 490; relative collision energy, 20%.

OHE$_1$), 2/4-OHE$_2$, 2/4-OHE$_3$] in the brain (Figure 3)[15]. It is well-known that the catechol estrogens are unstable; they are easily oxidatively degraded during their isolation and purification procedures and prone to be adsorbed into tissues. These features of the catechol estrogens have hampered the development of an assay for trace amounts of these compounds in a complex matrix such as the brain. We also spiked the authentic catechol estrogens in rat brains, extracted, treated with several solid phase cartridges, derivatized with silyl reagent and then subjected them to GC/MS, but these compounds were not detected. These data prompted us to derivatize the catechol estrogens to acetates (Ac) to prevent oxidation and adsorption and then subject them to detection using LC/APCI-MS[16].

### 4.1. LC/MS$^n$ of catechol estrogen acetates

Although the catechol estrogens (2/4-OHE$_2$) produced $[M+HCO_2]^-$ as the base peak in the LC/ESI-MS and $[M-H]^-$ in the LC/ESI-MS$^2$ (precursor ion, $[M+HCO_2]^-$ ; relative collision energy, 15%), no other molecular-related ions were obtained. On the other hand, it is expected that introducing the highly proton affinitive acetate group in the catechol estrogens gives more fragment ions which can be detected in positive-ion mode of LC/APCI-MS. As expected, the authentic catechol estrogen acetates (2/4-OHE$_1$diAc, 2/4-OHE$_2$triAc and 2/4-OHE$_3$tetraAc) produced $[M+NH_4]^+$ as the base peak and some characteristic molecular-related ions, such as $[M+H-42]^+$ and $[M-AcO]^+$, in the APCI-MS. LC/APCI-MS$^2$ (precursor ion, $[M+NH_4]^+$) also gave the $[M+H]^+$, $[M+H-42]^+$ and other product ions (Figure 4). It may be postulated that the *m/z* 42 reduced ions were formed by rearrangement of hydrogen and loss of ketene from the phenol acetates. Although a clear difference has not been observed in the mass spectra of the derivatives of the 2-hydroxyestrogens and 4-isomers, the above results were useful for the identification of the catechol estrogens in the brain.

### 4.2. Characterization of catechol estrogens in rat brains

The brains of adult male Wistar strain rats were homogenized in 10% EtOH containing ascorbic acid as an anti-oxidant and extracted with AcOEt, followed by derivatization with acetic anhydride-pyridine under mild conditions. After the purification with silica gel mini-column chromatography, ISOLUTE C18 (EC) and prep. HPLC, the obtained fractions corresponding to the catechol estrogen acetates were analyzed using LC/APCI-MS$^n$. These were identified in comparison with the authentic samples based on their chromatographic behavior during LC/APCI-MS$^n$, the mass spectra and the product ion mass spectra of these fractions (Figure 4). As a consequence, the existence of six catechol estrogens in the rat brains was

confirmed as acetates using LC/APCI-$MS^n$. The derivatization to acetate was effective in identifying the labile catechol estrogens in the biological substances. The development of the quantitative determination method for these estrogens in rat brains is now in progress in our laboratories.

## 5. Conclusions

The derivatization of PS and [$^2H_4$]-PS (IS) with DBD-H was useful for not only increasing the detection response but also protecting the deuterium-hydrogen exchange reaction of IS in LC/ESI-$MS^n$. Furthermore, the derivatization to acetate was useful for the treatment of labile catechol estrogens in the complex matrix and gave enough structural information in LC/APCI-$MS^n$.

These results suggest that this suitable derivatization method provides a more sensitive and selective analysis using LC/MS.

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