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Short communication

Enzymic conversion of 3β-hydroxy-5-ene-steroids and their sulfates to 3-oxo-4-ene-steroids for increasing sensitivity in LC–APCI-MS

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Abstract

A method of increasing the sensitivity of 3β-hydroxy-5-ene (Δ^5)-steroids in liquid chromatography–atmospheric pressure chemical ionization-mass spectrometry (LC–APCI-MS) based on the structural conversion by cholesterol oxidase (ChO) was demonstrated. The Δ^5-steroids were rapidly converted to their 3-oxo-4-ene (Δ^4)-forms by the treatment with ChO and the obtained Δ^4-forms provided 3–14-fold higher sensitivity compared to intact steroids in the positive-APCI-MS. This enzymic conversion method was also applied to the sulfated conjugates of Δ^5-steroids after solvolysis. The method enabled the detection of trace levels of dehydroepiandrosterone and androstenediol 3-sulfate in human serum, which could not be detected by the usual LC–MS.
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Keywords: Steroid; Cholesterol oxidase; Increasing sensitivity; LC–MS; Atmospheric pressure chemical ionization; Human serum

1. Introduction

Steroid hormones (androgens, gestogens, estrogens and corticoids) are essential biomolecules for the maintenance of life in human and animal organisms; they act as ligands for nuclear or intracellular receptors and have strong biological activity at very low concentrations (nanomolar and even picomolar) in target tissues. Except for estrogens, the steroid hormones have the 3-oxo-4-ene (Δ^4)-structure. On the contrary, numerous 3β-hydroxy-5-ene (Δ^5)-steroids including their sulfated conjugates are also present in a living body and are generally thought to be precursors or inactive metabolites of Δ^4-steroid hormones. However, several reports describe the relations between some Δ^5-steroids and diseases that frequently develop in the aged. For example, dehydroepiandrosterone (DHEA) is involved in the learning and memory performance as a neuroactive steroid, and its low serum concentration with aging may be a pathogenesis of dementia [1,2], and androstenediol 3-sulfate (Adiol-S) has been proposed as a biomarker of prostate cancer [3]. Therefore, the measurement of these Δ^5-steroids in biological fluids and tissues is useful for elucidation of the nature, diagnosis and treatment of these diseases.

Recently, liquid chromatography–mass spectrometry (LC–MS) has been widely used for steroid analysis due to its specificity and versatility [4]. However, the neutral Δ^5-steroids have a rather low response using either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) due to their low proton-affinitive or acidic properties. The sulfates are generally analyzed in the negative-ESI-MS due to the strong acidic property of the sulfonic group, but the actual sensitivity is usually lower than the expected one, because the ion separation on the capillary tip insufficiently proceeds in the negative-ion mode [5]. Thus, the conventional LC–MS sometimes does not demonstrate the required sensitivity for the trace analysis of the Δ^5-steroids. On the other hand, Δ^4-steroids show relatively higher responses in the positive-APCI-MS, because their protonated forms are stabilized due to the delocalization of the charge, i.e. resonance stabilization (Fig. 1).

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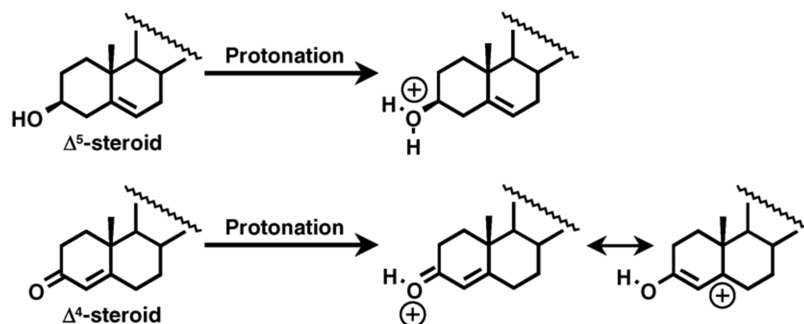

Fig. 1. Protonated forms of Δ^5- and Δ^4-steroids.

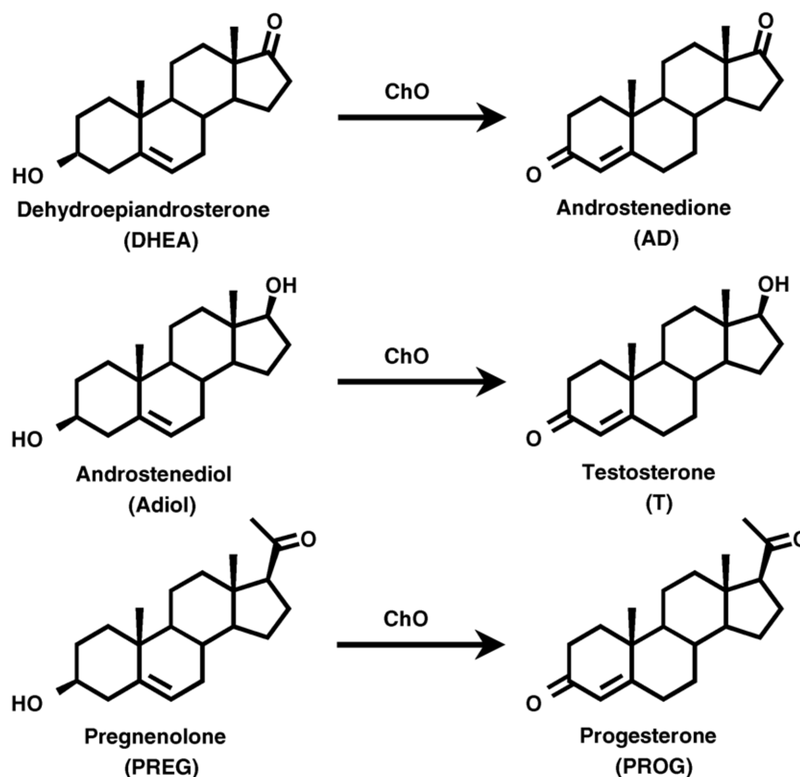

Fig. 2. Conversion of Δ^5-steroids to Δ^4-steroids with ChO.

Cholesterol oxidase (ChO) has a broad specificity and therefore can convert various Δ^5-steroids to their Δ^4-forms [6,7]. With this background information, we first examined the usefulness of the conversion of Δ^5-steroids to Δ^4-steroids with ChO for the detection responses in LC–APCI-MS (Fig. 2). Next, the application of the proposed enzymic conversion method to human serum assays was studied.

2. Experimental

2.1. Materials and chemicals

DHEA, androstenedione (AD), testosterone (T), pregnenolone (PREG) and progesterone (PROG) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Adiol and Adiol-S were prepared from DHEA and DHEA sulfate, respectively, by reduction with $NaBH_4$. PREG-S was synthesized in our laboratories by the known method [8]. Standard solutions of each steroid were prepared as 100 ng/ml in EtOH. ChO was obtained from Toyobo (Tokyo). Oasis HLB cartridges (60 mg adsorbent; Waters, Milford, MA, USA) were successively washed with AcOEt (2 ml), MeOH (2 ml) and H_2O (2 ml) prior to use. All other reagents and solvents were of analytical grade.

2.2. LC–MS

LC–MS was performed using a ThermoQuest LCQ (San Jose, CA, USA) connected to a JASCO PU-980 (Tokyo) chromatograph. The ZoomScan mode (one of the sensitive mass chromatographic modes of the LCQ mass spectrometer) was

used. The unconjugated steroids were analyzed using APCI-MS in the positive-ion mode: heated capillary temperature, 150 °C (Adiol) or 200 °C (other steroids); vaporizer temperature, 500 °C; sheath gas flow rate, 60 units; capillary voltage, 10 V and the tube lens offset, 15 V. A J'sphere ODS H-80 (4 μm, 150 mm × 4.6 mm i.d.; YMC, Kyoto, Japan) column was used at a flow rate of 1 ml/min at 40 °C. The sulfates were analyzed using ESI-MS in the negative-ion mode: heated capillary temperature, 280 °C; sheath gas flow rate, 60 units; auxiliary gas flow rate, 20 units; capillary voltage, −12 V and the tube lens offset, −20 V. A J'sphere ODS H-80 (4 μm, 150 mm × 2.0 mm i.d.) column was used at a flow rate of 0.15 ml/min at 40 °C.

2.3. ChO treatment of Δ^5-steroids

A mixture of standard Δ^5-steroids (1 ng) or a serum sample in EtOH (10 μl) and ChO (1 unit) in 50 mM Tris–HCl buffer (pH 7.6, 1 ml) was incubated in air at 37 °C for 20 min. The incubation specimen was mixed with MeOH (1 ml) and passed through an Oasis HLB cartridge. After washing with H_2O (2 ml) and 70% MeOH (2 ml), the steroids were eluted with AcOEt (1 ml), which was evaporated under an N_2 gas stream.

2.4. Acidic solvolysis of sulfates

The standard sulfates (1 ng) or a serum sample in EtOH (10 μl) was dissolved in AcOEt (0.5 ml) containing 2 μl of 0.5 M H_2SO_4. The mixture was stored at room temperature for 1 h, washed with H_2O (0.5 ml, twice), and the solvent was evaporated. The residue was dissolved in EtOH (10 μl) and subjected to the ChO treatment.

2.5. Effect of enzymic conversion for detection responses in Δ^5-steroid analysis

The effect of the conversion with ChO for the detection responses in Δ^5-steroid analysis was evaluated by the signal to noise ratio (S/N). The base ions of the respective steroids listed in Table 1 were monitored. Unconjugated Δ^5-steroids (1 ng) were treated with ChO and purified with an Oasis HLB cartridge as described above. The formed Δ^4-steroids were dissolved in EtOH (40 μl), and 10 μl of this solution was subjected to LC–APCI-MS. In like manner, the Δ^4-steroids formed from sulfates (1 ng) by solvolysis and the ChO treatment were dissolved in EtOH (40 μl), 10 μl of which was subjected to LC–APCI-MS. The S/N values obtained when ethanolic solutions of untreated Δ^5-steroids (unconjugated steroids or sulfates; 250 pg/10 μl) were subjected to LC–APCI-MS or LC–ESI-MS were used as the control values.

2.6. Pretreatment procedure for analysis of DHEA in human serum

Serum (100 μl) obtained from a healthy male subject (63 years) was added to MeCN (0.4 ml), vortex-mixed for 30 s and then centrifuged at 1500 × *g* (4 °C, 10 min). The solvent of the supernatant was evaporated, and the residue was dissolved in AcOEt (0.5 ml). After washing with H_2O (0.5 ml) and evaporation of the solvent, the residue was dissolved in EtOH (10 μl), treated with ChO, redissolved in EtOH (40 μl) and subjected to LC–APCI-MS (10 μl). In the case where DHEA was analyzed without the ChO treatment, the serum (100 μl) was deproteinized with MeCN (0.4 ml). The supernatant was diluted with Tris–HCl buffer (1 ml) and passed through an Oasis HLB cartridge. After washing with H_2O (2 ml) and 70% MeOH (2 ml), the steroid was eluted with AcOEt (1 ml), which was evaporated under an N_2 gas stream. The residue was dissolved in EtOH (40 μl), 10 μl of which was subjected to LC–APCI-MS analysis.

2.7. Pretreatment procedure for analysis of Adiol-S in human serum

Serum (20 μl) obtained from a healthy male subject (59 years) was added to MeCN (0.1 ml), vortex-mixed for 30 s

Table 1
Effect of enzymic conversion for detection responses in Δ^5-steroid analysis

Analyte/treatment (product)	Mobile phase[a]/t_R (min)	Monitoring ion (*m/z*)	S/N[b]	Increasing sensitivity
DHEA/without	7:3/6.5	271 $[M+H-H_2O]^+$	7.9	–
DHEA/ChO (AD)	2:1/5.9	287 $[M+H]^+$	33.2	4.2
Adiol/without	9:4/6.1	273 $[M+H-H_2O]^+$	2.8	–
Adiol/ChO (T)	2:1/6.9	289 $[M+H]^+$	39.5	14.1
PREG/without	4:1/6.1	299 $[M+H-H_2O]^+$	24.2	–
PREG/ChO (PROG)	7:2/5.6	315 $[M+H]^+$	71.9	3.0
Adiol-S/without	11:9/6.7	369 $[M-H]^-$	5.5	–
Adiol-S/solvolysis + ChO (T)	2:1/5.9	289 $[M+H]^+$	17.7	3.2
PREG-S/without	2:1/6.2	395 $[M-H]^-$	12.8	–
PREG-S/solvolysis + ChO (PROG)	7:2/5.6	315 $[M+H]^+$	19.5	1.5

[a] MeOH–H_2O (v/v) for unconjugated steroids, MeOH–10 mM $HCOONH_4$ (v/v) for sulfates.
[b] Without: Δ^5-steroids (1 ng) were dissolved in EtOH (40 μl), and 10 μl of this was then subjected to LC–MS. ChO: Δ^4-steroids formed from Δ^5-steroids (1 ng) by the ChO treatment were dissolved in EtOH (40 μl), and 10 μl of this was then subjected to LC–MS. Solvolysis + ChO: Δ^4-steroids formed from Δ^5-steroid sulfates (1 ng) by solvolysis and the ChO treatment were dissolved in EtOH (40 μl), and 10 μl of this was then subjected to LC–MS.

and centrifuged at 1500 × *g* (4 °C, 10 min). The solvent of the supernatant was evaporated, and the residue was dissolved in Tris–HCl buffer (0.5 ml) and passed through an Oasis HLB cartridge. After washing with H_2O (2 ml) and AcOEt (1 ml), the sulfate was eluted with MeOH (1.5 ml), which was evaporated under an N_2 gas stream. The residue was dissolved in EtOH (10 μl), solvolyzed, treated with ChO, redissolved in EtOH (40 μl) and subjected to LC–APCI-MS (10 μl). In the case where Adiol-S was analyzed without solvolysis and the ChO treatment, the eluent from the Oasis HLB cartridge was evaporated, and the residue was dissolved in MeOH–10 mM $HCOONH_4$ (11:9, v/v, 40 μl), 10 μl of which was subjected to LC–ESI-MS analysis.

3. Results and discussion

3.1. Effect of enzymic conversion for detection responses in Δ^5-steroid analysis

First, the detection responses of unconjugated Δ^5-steroids and their Δ^4-forms formed by the treatment with ChO in the positive-APCI-MS were compared, where the mobile phases were adjusted so that the retention time (t_R) values of the respective steroids were between 5.5 and 7 min. One nanogram of the Δ^5-steroids was incubated with ChO to convert into their Δ^4-forms and then purified with an Oasis HLB cartridge. To get the highest conversion efficiency, the amount of ChO (0.5, 1 and 2 units) and reaction time (10, 20, 40 and 60 min) were systematically examined. In consequence, it was found that Δ^5-steroids were quantitatively converted to their Δ^4-forms when they were incubated under the condition described in Section 2 (1 unit and 20 min). One quarter of the products was subjected to LC/MS; thus, the injection amounts of the products were apparently equivalent to 250 pg of the original steroids, if it was assumed that the conversion and recovery rates were both 100%. Based on this, the S/N values in the controls were determined by the injection of 250 pg of the intact Δ^5-steroids.

All the intact Δ^5-steroids provided $[M+H-H_2O]^+$ as the base ions together with a weak intensity of $[M+H]^+$, whereas the corresponding Δ^4-steroids gave essentially only $[M+H]^+$. The detection responses were improved several times by the ChO treatment of the unconjugated Δ^5-steroids as shown in Table 1. The effect of the treatment of Adiol was particularly significant; the treatment increased the sensitivity by 14-fold over the intact Adiol. Thus, the ChO treatment gave satisfactory results in increasing the sensitivity of Δ^5-steroids in APCI-MS as was expected.

Next, the application of the ChO treatment to the analysis of the sulfated Δ^5-steroids was examined (Table 1). The sulfates (1 ng) were solvolyzed and incubated with ChO to convert into their Δ^4-forms. Intact sulfates were analyzed by the negative-ESI-MS because they have a strong acidic functional group (sulfonic group). In the analysis of Adiol-S, the sample with solvolysis and the ChO treatment gave an obvious peak corresponding to T by positive-APCI-MS detection and its S/N value was 17.7, which was 3.2 times that in the analysis of intact Adiol-S in the negative-ESI-MS (S/N = 5.5). For PREG-S, solvolysis and the ChO treatment were also effective, but the magnitude of the increase of the sensitivity in the sulfated steroids (1.5–3.2 times) was

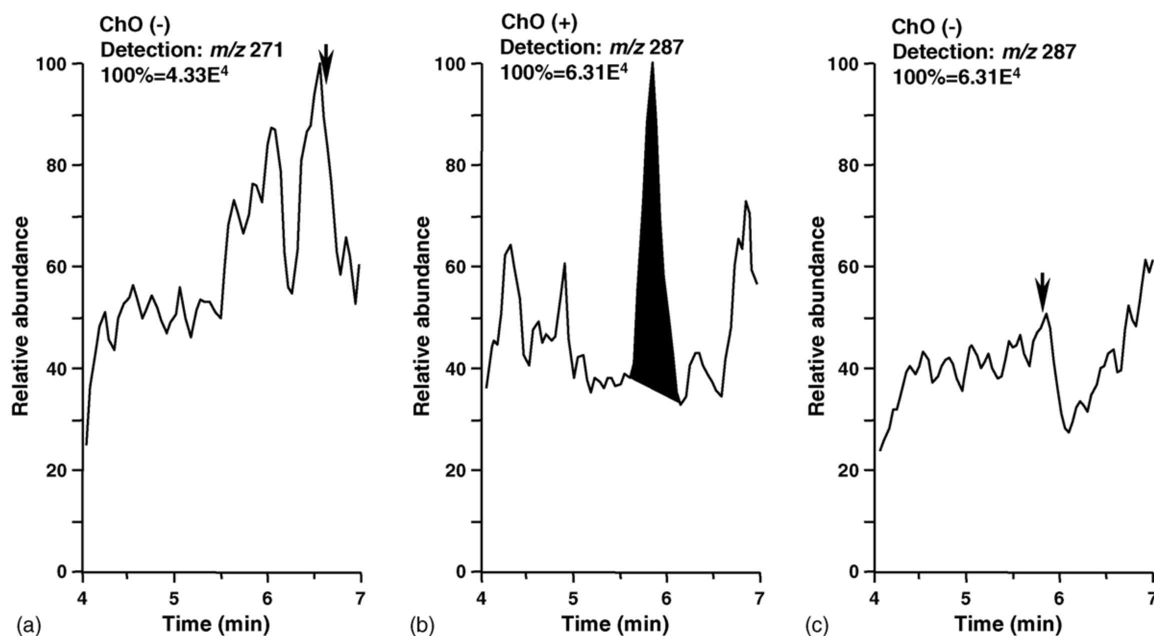

Fig. 3. Analysis of DHEA in human serum. (a) DHEA was analyzed without the ChO treatment. (b) DHEA was analyzed as AD after the ChO treatment. (c) The contamination of the endogenous AD was examined. All the samples were analyzed by the positive-APCI-MS. The arrows indicate the elution positions of DHEA (a) and AD (c).

generally not as good as that in the unconjugated steroids (3.0–14.1 times). This may be due to the low yield (about 40–50%) in the solvolysis of the sulfates. Although we are well aware that the present method has disadvantages in that information on the conjugation (position and form) is lost and the analytical procedure is more complicated than the direct analysis by ESI-MS, the method is sensitive and useful in the detection of a trace level of sulfates, such as Adiol-S.

3.2. Application of ChO treatment to analysis of Δ^5-steroids in human serum

To investigate the possibility of the proposed enzymic conversion method for biological sample analysis, unconjugated and sulfated Δ^5-steroids in human serum were analyzed. DHEA and Adiol-S were chosen as the target analytes, because they have been proposed as biomarkers of cognitive aging [1,2] and prostate cancer [3], respectively.

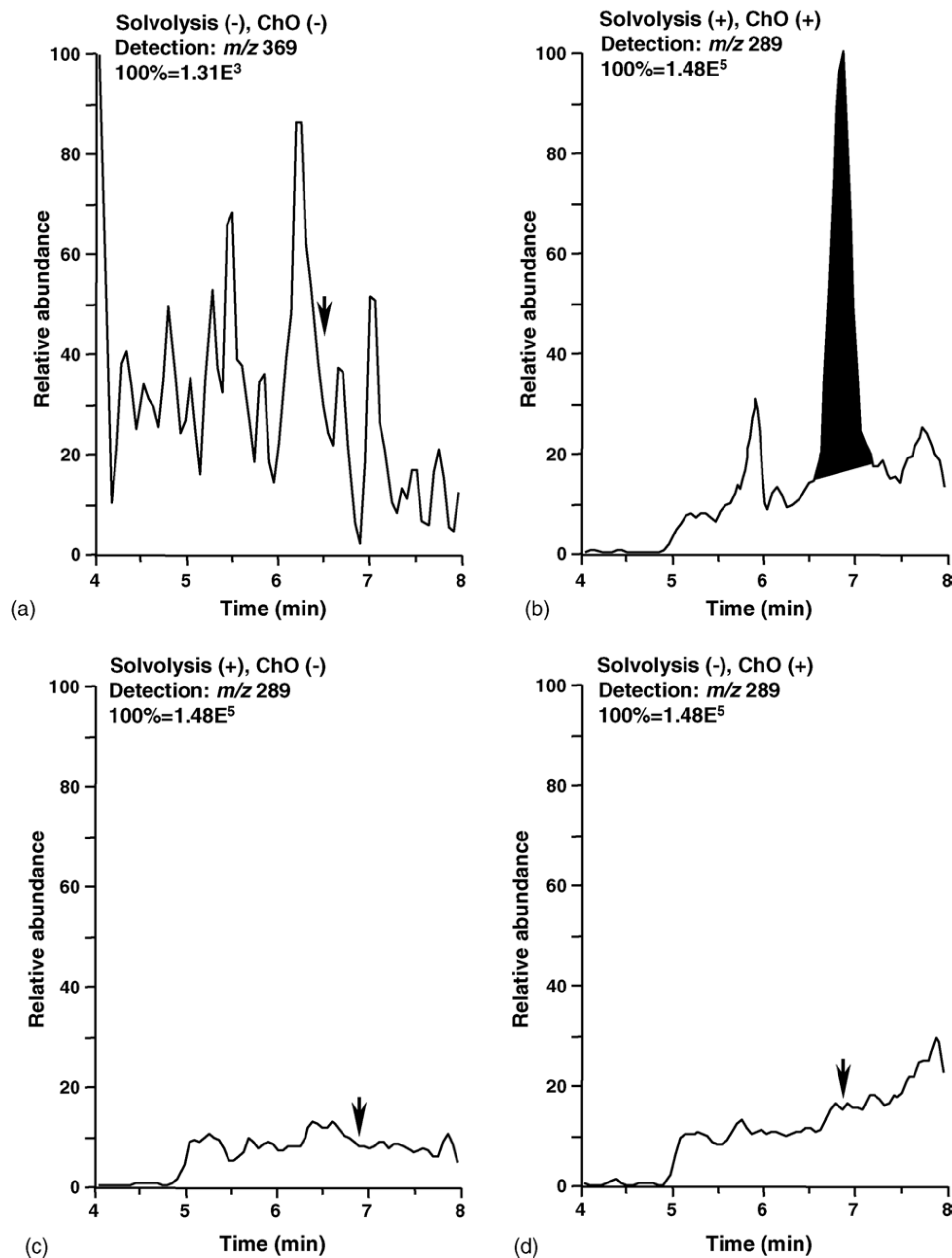

Fig. 4. Analysis of Adiol-S in human serum. (a) Adiol-S was analyzed without solvolysis and the ChO treatment by the negative-ESI-MS. (b) Adiol-S was analyzed as T after solvolysis and the ChO treatment. (c) The contamination of the endogenous T and its sulfate was examined. (d) The contamination of the endogenous Adiol was examined. The samples (b)–(d) were analyzed by the positive-APCI-MS. The arrows indicate the elution positions of Adiol-S (a) and T (c and d).

First, the analysis of DHEA by the proposed method was studied (Fig. 3). When the serum DHEA of a healthy male subject (63 years) was analyzed by LC–APCI-MS without the ChO treatment, the endogenous components disturbed the detection of DHEA (Fig. 3a). On the contrary, when DHEA was analyzed as AD after the treatment with ChO, the corresponding peak was clearly observed (Fig. 3b). From these results, the effect of the ChO treatment in increasing sensitivity was more significant in serum analysis than that observed in the standard sample analysis (4.2 times). The chromatogram obtained from the analysis of the serum AD in the sample without the ChO treatment is shown in Fig. 3c, in which AD was not detected; this result demonstrated that the peak observed in Fig. 3b was surely derived from DHEA without contamination of the endogenous AD. As the present method does not employ the separation procedure of unconjugated Δ^5-steroids from their Δ^4-forms, it can be applied to only the case where the serum level of the Δ^5-steroid is relatively higher than that of its Δ^4-form. However, this method is useful for the analysis of the serum Δ^5-steroids, such as DHEA, as demonstrated here.

Next, Adiol-S in the serum obtained from a healthy male subject (59 years) was analyzed. The serum (20 μl) was fractionated with an Oasis HLB cartridge, where the unconjugated steroids were selectively washed out with AcOEt, and the desired sulfate was then quantitatively recovered with MeOH [9]. The eluent was directly analyzed by the previously reported LC–ESI-MS [3], but Adiol-S was not detected due to the lack of sensitivity (Fig. 4a). The limit of detection in the LC–ESI-MS method for the serum Adiol-S was 250 pg per injection (S/N = 5), and 100 μl of sample was required for its quantification [3]. When the above eluent from the cartridge was solvolyzed, treated with ChO and then analyzed as T by LC–APCI-MS, the corresponding peak was surely observed (Fig. 4b). The contamination of the endogenous T and its sulfate was examined with the sample without the ChO treatment, and it was not detected (Fig. 4c). Furthermore, in the sample without solvolysis, the peak corresponding to T, which was derived from endogenous Adiol, was also not observed (Fig. 4d). These data show that the method can detect a trace level of Adiol-S without interference of endogenous components including unconjugated steroids.

4. Conclusion

The present study has demonstrated the significant sensitivity increase of Δ^5-steroids analyzed by LC–APCI-MS following the treatment with ChO. The structural conversion of Δ^5-steroids with ChO increased their detectabilities in the positive-APCI-MS and was also applicable to the analysis of sulfates when combined with solvolysis. The developed method also gave satisfactory results in serum analysis; the method enabled the detection of trace levels of DHEA and Adiol-S in human serum, which could not be detected by the usual LC–MS. Furthermore, it is expected that the method can be used for the quantitative analysis of Δ^5-steroids in biological samples by using suitable internal standards, such as deuterium-labeled steroids. Such studies are now in progress in our laboratories.

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