Fusaric Acid as a Novel Proton-Affinitive Derivatizing Reagent for Highly Sensitive Quantification of Hydroxysteroids by LC-ESI-MS/MS

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A highly sensitive derivatization method for liquid chromatography (LC)-electrospray ionization (ESI) tandem mass spectrometry of dehydroepiandrosterone (DHEA), testosterone (T), pregnenolone (P5), and 17 α -OH-pregnenolone (17-OHP5) was developed based on the use of fusaric acid as a reagent. DHEA, P5, and 17-OHP5 were rapidly and quantitatively converted to the 3-fusarate esters by treatment with fusaric acid and 2-methyl-6-nitrobenzoic anhydride. The positive ESI-mass spectra of the fusarate esters of each steroid were dominated by the appearance of $[M + H]^+$ as base peaks. The fusarate derivatization of these steroids showed 17.6-fold (DHEA), 11.9-fold (P5), 3.3-fold (17-OHP5), and 1.8-fold (T) higher sensitivity to those of the corresponding picolinate derivatives in LC-selected reaction monitoring. (J Am Soc Mass Spectrom 2010, 21, 249–253) © 2010 American Society for Mass Spectrometry

In the recent development of analytical methodology for hydroxysteroids that were generally insensitive to liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), two types of derivatization designs have been proposed to provide high sensitivity to such kind of steroid molecules. The introduction of permanently charged moieties into steroid molecules was proven to be a promising method [1–4]. The use of 1-methylpyridinium for hydroxy group [2] and 2-hydrazino-1-methylpyridine for carbonyl group [4] as derivatizing reagents showed higher sensitivity in analyses of testosterone (T), 5 α -dihydrotestosterone (DHT), and dehydroepiandrosterone (DHEA) by LC-ESI-MS/MS. However, the application of these permanently charged derivatives to steroids possessing more than two hydroxy and/or carbonyl groups in one molecule seemed to be inconvenient and failed to obtain simple ESI-mass spectra with the appearance of protonated molecule ($[M + H]^+$) due to the formation of multiple ions of $[M - H]^-$ and $[M]^{2+}$ [4].

The introduction of high proton-affinitive moiety into steroid molecules was the alternative choice to obtain simple ESI mass spectra characterized by the formation of $[M + H]^+$ with high intensity [5–8]. The use of dimethylaminoaphthalene-sulfonate [5, 6], pyridine-sulfonate [7], pyridine-dicarboxylate [8] deriva-

tives for steroidal hydroxyl group, and hydroxime [9, 10] and 2-pyridine-hydrazone [11] derivatives for steroidal ketones have been investigated. However, the introduction of sulfonate moieties was limited to the phenolic hydroxy group of estrogens. Derivatization of T and DHT with pyridine-2,3-dicarboxylic anhydride resulted in the formation of the mixture consisted of two regio-isomers of half esters in their chromatograms [8]. Generation of *syn*- and *ant*-isomeric peaks might also be inevitable in the analyses of T and DHT using hydroxime and pyridine-hydrazone derivatizations [9, 11].

Recently, we have demonstrated that the introduction of picolinate moiety into hydroxysteroids provided some advantages to LC-ESI-MS/MS analyses of these compounds in a positive mode, involving the production of simple $[M + H]^+$ with high intensity, the simplicity of the derivatization and purification process [12–14], and the excellence for liquid chromatographic behaviors in the profile analyses of corticosteroids [15–17] and sterols [18–20]. In our preliminary results, the picolinate derivatives of Δ^5 -3 β -hydroxysteroids, such as DHEA and pregnenolone (P5), seemed to be less sensitive in ESI compared with those of T and estradiol by selected reaction monitoring (SRM). This report describes the characteristics of fusaric acid derivative, as a novel and sensitive proton-affinitive tag for hydroxysteroids in LC-ESI-MS/MS, and presents some possible explanations to the enhancement of detection sensitivity by this derivative in LC-ESI-MS/MS compared with the picolinic acid derivative.

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Experimental

Materials and Reagents

Dehydroepiandrosterone (DHEA, **1a**), testosterone (T, **2a**), pregnenolone (P5, **3a**), and 17 α -hydroxypregnenolone (17-OHP5, **4a**) were purchased from Steraloids (Newport, RI, USA). Fusaric acid was obtained from Sigma-Aldrich (St. Louis, MO, USA), and 2-methyl-6-nitrobenzoic anhydride, 4-dimethylaminopyridine, and triethylamine were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). LC-MS grade ultra pure water, acetonitrile (MeCN), and methanol (MeOH) were purchased from Wako Pure Chemicals (Osaka, Japan). Oasis HLB cartridge (30 mg, 1 mL) was obtained from Waters (Milford, MA, USA).

LC-ESI-MS/MS

LC-ESI-MS/MS was run on a Finnigan TSQ Quantum triple-stage quadrupole mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA) equipped with an ESI ion source and a Surveyor auto-sampler and a MS-pump (Thermo Fischer Scientific). The columns was a Puresil 5-C₁₈ (150 mm $\times$ 2.1 mm i.d., 5 μ m, Waters) connected to a guard cartridge (C₁₈, 10 mm $\times$ 2 mm i.d., 5 μ m) in a guard-cartridge holder with the mobile phase consisted of MeCN-ultrapure water-acetic acid (AcOH) (85:15:0.1, vol/vol/vol) at a flow rate of 200 μ L/min. Elution was performed with an isocratic mode and at an ambient temperature. The general ESI-MS conditions were as follows: spray voltage, 4500 V (positive); sheath gas, nitrogen, 30 arbitrary unit (gas pressure); auxiliary gas, nitrogen, 15 arbitrary unit (gas pressure); ion transfer capillary temperature, 350 $^{\circ}$ C; collision gas argon, 1.5 mTorr (gas pressure).

Synthesis of Standard Derivatives

To a solution of DHEA (**1a**, 50 mg; 0.17 mmol in tetrahydrofuran (THF) 1 mL) was added the premixed reagent solution (fusaric acid; 40 mg, 2-methyl-6-nitrobenzoic anhydride; 50 mg, 4-dimethylaminopyridine; 10 mg, triethylamine; 50 μ L, THF 1 mL), and the reaction mixture was stirred at room temperature for 1 h. After dilution of the reaction mixture with 5% NaHCO₃ (20 mL), the product was then extracted with ethyl acetate EtOAc (20 mL). The organic layer was washed with sat. NaCl (20 mL), 5% HCl (20 mL), and then with sat. NaCl (20 mL $\times$ 2), and dried over anhydrous Na₂SO₄. Evaporation of the solvent and re-crystallization of the crude product from EtOAc afforded compound **1c** as colorless leaflets. Similarly, **2c** (colorless prisms), **3c** (colorless needles), and **4c** (colorless prisms) were prepared from **2a**, **3a**, and **4a**, respectively. Picolinate derivatives (**3b**: colorless needles and **4b**: colorless needles) were synthesized in the same manner as **1b** and **2b**, and the structures of all derivatives were confirmed by the measurement of ¹H nuclear magnetic resonance spectra, ultra violet spectra, and elemental analyses as previously described [12].

Microscale Derivatization [13, 14]

To a solution of steroid (**1a**, **2a**, **3a**, or **4a**; 1 ng or 1 μ g in THF 50 μ L) were added the premixed reagent solution (20 μ L) (fusaric acid; 40 mg, 2-methyl-6-nitrobenzoic anhydride; 50 mg, 4-dimethylaminopyridine; 10 mg, triethylamine; 100 μ L in THF 1 mL), and the resulting mixture was allowed to stand at room temperature for 30 min. After dilution of the reaction mixture with 5% NaHCO₃ (1 mL), the resulting sample was transferred onto Oasis HLB cartridge (30 mg-1 mL, preconditioned with MeOH 1 mL and ultra pure water 1 mL). The cartridge was washed with ultra pure water (3 mL), 5% HCl (3 mL), and ultra pure water (3 mL), and the derivative was eluted with 90% MeCN (1 mL). The eluate was evaporated to dryness under reduced pressure and the residue was dissolved in mobile phase (100 μ L) and then submitted to LC-ESI-MS/MS. Reaction yield was calculated by comparing the peak intensity prepared in this section with that of the sample obtained and purified in the former section (*Synthesis of standard*) by SRM.

Results and Discussion

As shown in Figure 1, the fusaric acid moiety was incorporated into the steroid molecule via 3 β -hydroxy

Figure 1. Schematic presentation of the derivatization of DHEA (**1a**), T (**2a**), P5 (**3a**) and 17-OHP5 (**4a**) with picolinic acid and fusaric acid by mixed anhydride method using 2-methyl-6-nitrobenzoic anhydride.

group (**1a**, **3a**, **4a**) or 17 β -hydroxy group (**2a**) by ester formation with mixed anhydride method using 2-methyl-6-nitrobenzoic anhydride [21] and 4-dimethylaminopyridine. Fusarate formation by this method underwent quantitatively under mild condition with reaction yield of >85% for **1c**, **2c**, **3c**, and **4c** in the levels of 1 ng and 1 μ g as was observed in the picolinate derivatization [13, 14].

Figure 2 shows the positive ESI mass spectrum (a) and the product ion spectrum (b) of the fusarate derivative of DHEA (**1c**). Positive-ESI-mass spectrum of **1c** was dominated by the appearance of protonated molecule ($[M + H]^+$) at m/z 450 as a base peak, and the formation of adduct ions ($[M + Na + CH_3CN]^+$) was observed at m/z 513. Collision of $[M + H]^+$ with argon (collision energy: 21 V) resulted in the formation of product ions at m/z 271 and 253, these ions were assigned to be formed by the elimination of fusaric acid ($C_{10}H_{13}NO_2$) moiety followed by the additional dehydration from $[M + H]^+$ in **1c**. The formation of these kinds of product ions is not specific to the fusarate derivatives, and is commonly observed in the picolinate derivatives and other pyridine-carboxylate derivatives of **1a** and **2a** as previously reported [12, 14]. Table 1 summarizes the positive-ESI mass and product ion spectral data of these four fusarate derivatives (**1c**, **2c**, **3c**, and **4c**). The same product ion species were observed at m/z 271 and 253 in **2c**, m/z 299 and 281 in **3c**, and m/z 315 and 297 in **4c**, respectively. The formation of product ion at m/z 180 originated from the fusaric acid moiety was also observed in all cases, and dominated particularly in **2c**.

In SRM, the transitions of m/z 450 $\rightarrow$ m/z 271 (or m/z 253) for **1c** and **2c**, m/z 478 $\rightarrow$ m/z 281 for **3c** and m/z 494 $\rightarrow$ m/z 297 for **4c**, were chosen, respectively, and detection sensitivities were compared with those of the picolinate derivatives (**1b**, **2b**, **3b**, and **4b**). The results are shown in Table 1 (right column). When the detection sensitivity was evaluated by measuring the signal-to-noise (SN) ratios for each peak of 20 fg (injected amount), the values of **1c**, **2c**, **3c**, and **4c** were >60, 2, 15, and 8, respectively. These SN ratios were higher to those of the corresponding picolinate derivatives. The increase of SN ratio was remarkable in **1c**, **3c**, and **4c** rather than **2c**. When absolute detection responses of the fusarate derivatives were compared with those of the corresponding picolinate derivatives by SRM (0.1 pmol; injected amount), the fusarate derivatives of each steroid exhibited higher responses than those of the picolinate derivatives. The fusarate derivative of T (**2c**) showed 1.8 ± 0.2 (mean $\pm$ standard deviation; $n = 3$)-fold higher response to that of **2b**, whereas, the fusarate derivatives of DHEA (**1c**), P5 (**3c**), and 17-OHP5 (**4c**) showed 17.6 ± 3.0 -fold, 11.9 ± 2.4 -fold, and 3.3 ± 1.0 -fold higher responses when compared with those of **1b**, **3b**, and **4b**, respectively. The typical SRM chromatograms of these steroids in matrices is explained in the Supplementary Material, which can be found in the electronic version of this article.

As judged from these results, the derivatization of steroidal hydroxy group by fusaric acid must be a promising technique to afford an excellent sensitivity to the assay of DHEA, P5, and 17-OHP5 by LC-ESI-MS/MS. Appearance of the differences in SRM sensitivity

Figure 2. Positive ESI mass spectrum (a) and product ion spectrum (b) of the fusarate derivative of DHEA (**1c**). Spray voltage: 4500 V.

Table 1. ESI-mass spectral, tandem mass spectral data and signal-to-noise (SN) ratios in SRM of the fusarate derivatives of DHEA (1c), T (2c), P5 (3c), and 17-OHP5 (4c)

Derivatives	ESI-MS data: <i>m/z</i> (intensity, %)		MS/MS data: product ions <i>m/z</i> (collision energy; V)			SN ratios (20 fg/on column) ^a
	[M + H] ⁺	Other ions				
1c	450 (100)	513 ^b (35), 271 ^c (28)	271 (21)	253 (28)	180 (21)	>20 ^d , >60 ^e
2c	450 (100)	513 ^b (16), 271 ^c (1) ^e	180 (20)	253 (27)	271 (22)	2 ^d
3c	478 (100)	541 ^b (21), 299 ^c (22)	299 (16)	281 (21)	180 (18)	15 ^f
4c	494 (100)	557 ^b (18), 315 ^c (50)	297 (21)	315 (19)	180 (19)	8 ^g

^aValues were expressed as injected amounts.^b[M + Na + CH₃CN]⁺.^c[M + H – C₁₀H₁₃O₂N(fusaric acid) – H₂O]⁺.^d*m/z* 450 → *m/z* 253 (27 V).^e*m/z* 450 → *m/z* 271 (21 V).^f*m/z* 478 → *m/z* 281 (21 V).^g*m/z* 494 → *m/z* 297 (21 V).

between the fusarate derivatives and picolinate derivatives for these hydroxysteroid partially are supposed to be attributed to the theoretically higher value of the proton-affinity of 5-butylpyridine (fusarate) moiety (ca 229–230 kcal/mol, estimated value from the database; <http://webbook.nist.gov/chemistry/> of National Institute of Standard and Technology (NIST)) than that of pyridine moiety (ca 222.4 kcal/mol). The reason for the observation of difference in the enhancement of sensitivity between T and DHEA was not made clear at this moment.

Conclusions

Derivatization of hydroxysteroids with fusaric acid and the characterization of the prepared derivatives in LC-ESI-MS/MS (positive mode) were described. The use of fusarate derivatization for Δ⁵-3β-hydroxysteroids provided robustness and enhanced sensitivity to the assay, and enabled to determine these steroids with the levels of less than sub-femtogram on column. Improvement of sensitivity of fusarate derivative in LC-ESI-MS/MS might partially be attributed to the inherent high proton-affinity of fusarate moiety than that of the picolinate moiety.

Acknowledgments

The authors acknowledge support in part for this work by a High Technology Research Center Project from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at [doi:10.1016/j.jasms.2009.10.008](https://doi.org/10.1016/j.jasms.2009.10.008).

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