Alternative Procedure for Charged Derivatization to Enhance Detection Responses of Steroids in Electrospray Ionization-MS

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A derivatization procedure has been examined to enhance the electrospray ionization (ESI)-MS detectabilities of steroids that charged derivatization is not suitable for. The derivatization procedure with 2-hydrazinopyridine or isonicotinoyl azide was very effective for the sensitive detection of di-oxosteroids or di-hydroxysteroids, respectively, and the detection limits of the resulting derivatives were as low as about 2 fmol. The derivatives also provided intense characteristic product ions in the MS–MS, which are expected to be usable for the selected reaction monitoring mode.

Key words derivatization; electrospray ionization (ESI)-MS; steroid; detection response

Steroids exert strong biological activities at very low concentrations (nanomolar and even picomolar) *via* their specific intracellular/nuclear receptors in the target organs. Some steroids referred to as neuroactive steroids also affect neurotransmission through action at the membrane neurotransmitter receptors in the nervous system.[1] A specific and sensitive method for the characterization and determination of steroids in biological fluids and tissues is useful for the elucidation of the nature, diagnosis and treatment of diseases. Numerous methods have been described to analyze steroids, such as immunoassay, receptor binding assay, HPLC and GC–MS,[2] but every one of these approaches has both advantages and disadvantages.

Liquid chromatography (LC) coupled with electrospray ionization (ESI)-MS is now widely being used for steroid analysis due to its specificity, versatility and simultaneous multi-analyte quantification capability.[3,4] Steroids with the 3-oxo-4-ene-structure, such as testosterone (T), can be detected at the low-picogram level by the positive ESI-MS in the selected ion monitoring (SIM) or selected reaction monitoring (SRM) mode, because they are more basic than most neutral steroids due to the delocalization of the charge (*i.e.*, resonance stabilization) in the protonated form and can provide some characteristic A-ring product ions in MS–MS.[5,6] However, the ionization efficiencies of steroids without the 3-oxo-4-ene-structure (*i.e.*, 5-ene-steroids, 5α/β-reduced-steroids and estrogens) are relatively low for ESI; conventional LC–ESI-MS(–MS) sometimes does not demonstrate the required sensitivity for the trace analysis of these steroids.

Because the ionization process occurs in the liquid-phase during ESI, the pre-formation of ions is very important in the ESI detection mode. Based on this, the introduction of permanently charged moieties by derivatization (*i.e.*, charged derivatization) has been successfully used for increasing the detection responses of poorly ionizable steroids during ESI-MS.[7—11] These charged derivatives can readily provide an intense molecular cation, $[M]^+$, in the positive ESI-MS. However, we found that tagging two charged groups to steroids

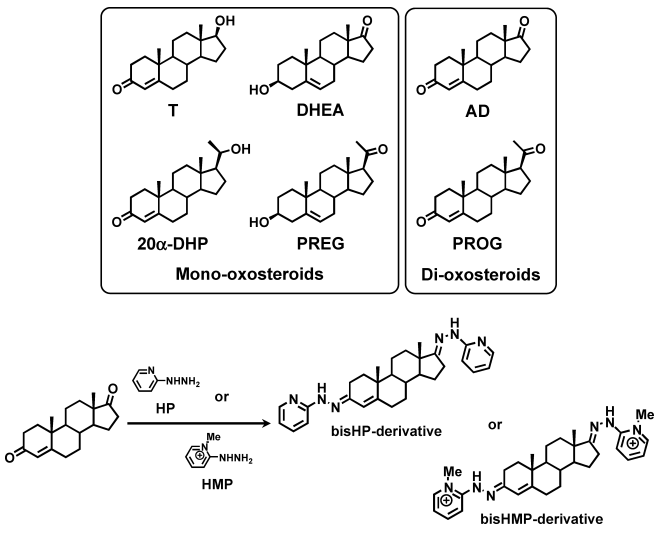

Fig. 1. Chemical Structures of Oxosteroids Examined in This Study and Their Derivatization Procedures

Derivatization of AD is an example.

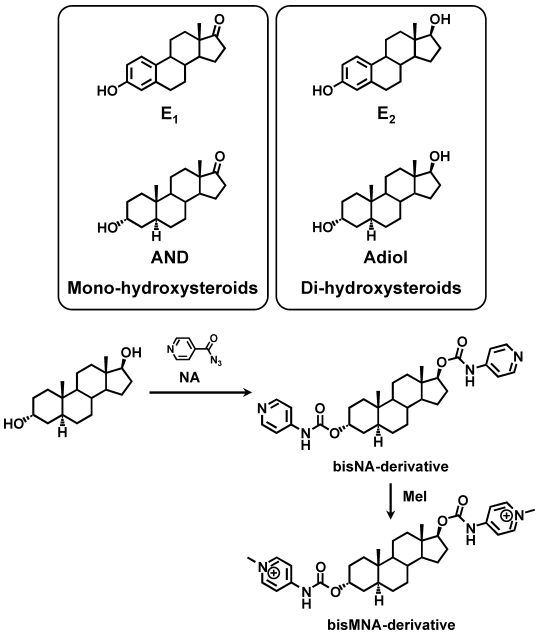

Fig. 2. Chemical Structures of Hydroxysteroids Examined in This Study and Their Derivatization Procedures

Derivatization of Adiol is an example.

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having two functional groups, such as androstenedione and progesterone, is not effective for increasing their detection responses. It seemed that this phenomenon occurred due to the fact that the small molecules with a multi-charge are unstable in the gas-phase, because the above derivatives provided a multiple number of ions, $[M-1]^+$, $[M]^{2+}$ and fragment ions, instead of $[M]^+$.[12]

With this background information, we examined an alternative derivatization procedure that can improve the detection responses of steroids, for which the currently used charged derivatization is not effective. In this paper, we describe the usefulness of the introduction of the pyridyl group, that is not a permanently charged moiety but highly proton-affinitive one, for the ESI-MS detection of steroids having two of the same functional groups (Figs. 1, 2).

Experimental

Materials and Chemicals T, dehydroepiandrosterone (DHEA), androstenedione (AD), pregnenolone (PREG), progesterone (PROG), estrone (E_1), estradiol (E_2), androsterone (AND) and 2-hydrazinopyridine (HP) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). 20α-Dihydroprogesterone (20α-DHP) and 5α-androstane-3α,17β-diol (Adiol) were obtained from Steraloids (Newport, RI, U.S.A.). The stock solutions of each steroid were prepared as 100 μg/ml solutions in ethanol and subsequent dilutions were carried out using ethanol. 2-Hydrazino-1-methylpyridine (HMP) was synthesized in our laboratories as previously reported.[12] Isonicotinoyl azide (NA) was prepared from isonicotinic acid (Tokyo Kasei Kogyo) by the known method.[13] All other reagents and solvents were of analytical grade.

Derivatization of Oxosteroids with HP or HMP To a solution of oxosteroid in ethanol (30 μl), a freshly prepared solution of HP or HMP (10 μg) in ethanol (50 μl) containing 25 μg of trifluoroacetic acid was added, and the mixture was maintained at 60 °C for 1 h. After removal of the solvents, the product was dissolved in methanol–10 mM ammonium formate (1 : 1, v/v), an aliquot of which was subjected to LC–MS(–MS).

Derivatization of Hydroxysteroids with NA To a hydroxysteroid, a freshly prepared solution of NA (40 μg) in benzene (40 μl) was added, and the mixture was maintained at 80 °C for 30 min. After the further addition of the reagent (40 μg in benzene 40 μl), the entire mixture was further maintained at 80 °C for 30 min. After the addition of ethanol (500 μl), the solvents were evaporated. The product was then dissolved in methanol–10 mM ammonium formate (1 : 1, v/v), an aliquot of which was subjected to LC–MS(–MS).

Quaternarization of NA Derivatives To an NA derivative prepared as described above, methyl iodide (100 μl) was added. The mixture was maintained at 60 °C for 15 min, and then the excess reagent was evaporated off. The methylated NA (MNA) derivative was dissolved in methanol–10 mM ammonium formate (1 : 1, v/v), an aliquot of which was subjected to LC–MS(–MS).

LC–MS(–MS) LC–MS(–MS) was performed using an Applied Biosystems API 2000 triple stage quadrupole-mass spectrometer (Foster City, CA, U.S.A.) connected to a Shimadzu LC-10AT chromatograph (Kyoto, Japan). A YMC-Pack Pro C18 RS (5 μm, 150×2.0 mm i.d.; YMC, Kyoto) was used at the flow rate of 0.2 ml/min at 40 °C. All the compounds were analyzed by ESI-MS in the positive-ion mode and the conditions were as follows: declustering potential; 30 V (intact steroids) or 80 V (the HMP, HP, NA and MNA derivatives), focusing potential; 380 V (intact steroids) or 200 V (the HMP, HP, NA and MNA derivatives), entrance potential; 10 V, ion spray voltage, 5 kV, curtain gas; 45 psi, ion source gas 1; 80 psi, ion source gas 2; 80 psi, turbo gas temperature; 500 °C and interface heater; on. Nitrogen was used as the collision gas in the MS–MS experiment with the collision energy of 35—55 eV. The data were collected and quantified using Applied Biosystems Analyst software (version 1.3.1). The smoothing options were as follows: default number of smooths, 5; previous point weight, 0.5; current point weight, 1 and next point weight, 0.5.

Effect of Derivatization for Detection Responses The effect of the derivatization for the detection responses was evaluated by the limit of detection [LOD; the amount of intact steroids or derivatives per injection giving a signal to noise ratio (S/N) of 5]. The S/N values were manually calculated by division of the peak height of a target steroid by the noise level around the peak. The base ions listed in Table 1 were monitored in the SIM mode. The steroids (20 or 100 pg) were converted to their HP, HMP, NA or MNA derivatives as described above. These derivatives were dissolved in methanol–10 mM ammonium formate (1 : 1, v/v, 100 μl) and then subjected to LC–MS. By stepwise decreasing the injection volume of the resulting solution, the amount of derivative giving an S/N of 5 was determined. The LODs of the intact steroids were determined using a solution of 1 ng/ml or 100 ng/ml in the same way.

Results and Discussion

ESI-MS of Derivatives For the ESI-MS operating in the positive-ion mode, all the HP and NA derivatives provided their protonated molecules, $[M+H]^+$ as the base peak ions, regardless of the number of introduced pyridyl group (Tables 1, 2 and Fig. 3a; the ESI-MS spectrum of the bisNA derivative of Adiol is an example). This was completely different from the ionization behavior of the HMP or MNA derivative; although the HMP derivatives of the mono-oxosteroids (T, DHEA, 20α-DHP and PREG) and the MNA derivatives of the mono-hydroxysteroids (E_1 and AND) provided only their molecular cations, the bisHMP (AD and PROG) and bisMNA (E_2 and Adiol) derivatives gave $[M-1]^+$ as the base peak ions together with some fragment ions. For the bisHMP derivatives, $[M]^{2+}$ was also observed.

Effect of Derivatization for Detection Responses The effects of the respective derivatizations for the detection responses were evaluated by the LODs, in which the mobile phases were adjusted so that the t_R values of the respective steroid derivatives were around 6 min (when a derivative gave twin peaks due to the *E*- and *Z*-isomers, its major peak was used for this study) (Tables 1, 2).

Although both the HP and HMP derivatives of the mono-

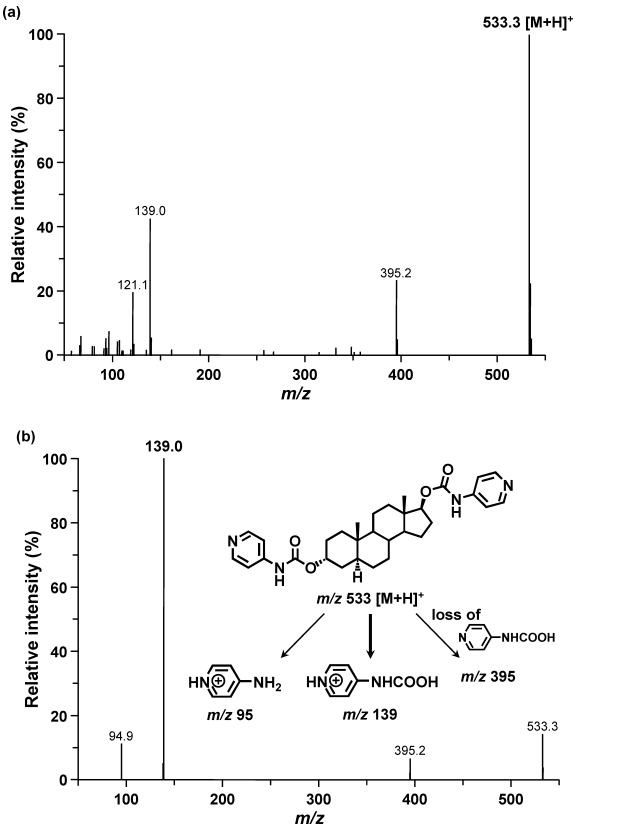

Fig. 3. ESI-MS (a) and ESI-MS–MS (b) Spectra of bisNA Derivative of Adiol

Adiol (100 ng) was derivatized and then dissolved in methanol–10 mM ammonium formate (1 : 1, v/v, 100 μl), 2 μl of which was subjected to LC–MS(–MS).

Table 1. ESI-MS Data of Intact Oxosteroids and Their Derivatives

Compound (mw)	Base peak ion (*m*/*z*) (Monitoring ion)	Mobile phase[a] [t_R (min)]	LOD (fmol)	Increasing sensitivity[b]
T (intact) (288)	289.3 $[M+H]^+$	3 : 1 (5.9)	14 (4.0)[c]	1
T-HP (379)	380.2 $[M+H]^+$	6 : 1 [(4.4)[d], 5.5]	2.4 (0.7)	5.8
T-HMP (394)	394.2 $[M]^+$	3 : 1 [(4.6)[d], 5.7]	1.0 (0.3)	14
DHEA (intact) (288)	271.2 $[M+H-H_2O]^+$	5 : 2 (5.7)	2800 (800)	1
DHEA-HP (379)	380.2 $[M+H]^+$	5 : 1 (5.8)	1.7 (0.5)	1600
DHEA-HMP (394)	394.1 $[M]^+$	2 : 1 (5.6)	1.7 (0.5)	1600
AD (intact) (286)	287.2 $[M+H]^+$	3 : 1 (5.5)	18 (5.0)	1
AD-bisHP (468)	469.3 $[M+H]^+$	10 : 1 (6.5)	2.1 (0.6)	8.6
AD-bisHMP (498)	497.4 $[M-1]^+$	3 : 1 [(5.0)[d], 5.6]	11 (3.0)	1.6
20α-DHP (intact) (316)	317.2 $[M+H]^+$	7 : 2 (5.8)	13 (4.0)	1
20α-DHP-HP (407)	408.3 $[M+H]^+$	6 : 1 [(4.7)[d], 6.4]	2.8 (0.9)	4.6
20α-DHP-HMP (422)	422.4 $[M]^+$	9 : 2 [(5.0)[d], 5.8]	1.3 (0.4)	10
PREG (intact) (316)	317.2 $[M+H]^+$	7 : 2 (6.2)	950 (300)	1
PREG-HP (407)	408.3 $[M+H]^+$	15 : 2 (5.8)	1.9 (0.6)	500
PREG-HMP (422)	422.3 $[M]^+$	4 : 1 (5.6)	1.3 (0.4)	730
PROG (intact) (314)	315.0 $[M+H]^+$	10 : 3 (5.6)	6.4 (2.0)	1
PROG-bisHP (496)	497.4 $[M+H]^+$	20 : 1 [(5.5)[d], 6.3]	2.2 (0.7)	2.9
PROG-bisHMP (526)	525.2 $[M-1]^+$	13 : 2 (5.6)	11 (3.5)	0.6

a) The proportions (v/v) of methanol–10 mM ammonium formate. *b*) The sensitivities of intact steroids are taken as 1. *c*) The values in parentheses are amounts (pg) converted into intact steroids. *d*) The values in parentheses are t_Rs of the minor peaks of *E*- and *Z*-isomers.

Table 2. ESI-MS Data of Intact Hydroxysteroids and Their Derivatives

Compound (mw)	Base peak ion (*m*/*z*) (Monitoring ion)	Mobile phase[a] [t_R (min)]	LOD (fmol)	Increasing sensitivity[b]
E_1 (intact) (270)	271.2 $[M+H]^+$	2 : 1 (5.7)	1300 (350)[c]	1
E_1-NA (390)	391.2 $[M+H]^+$	9 : 4 (6.2)	5.6 (1.5)	230
E_1-MNA (405)	405.1 $[M]^+$	9 : 10 (6.4)	3.0 (0.8)	430
E_2 (intact) (272)	273.3 $[M+H]^+$	2 : 1 (5.6)	2400 (650)[c]	1
E_2-bisNA (512)	513.2 $[M+H]^+$	3 : 2 (6.2)	4.0 (1.1)	600
E_2-bisMNA (542)	541.1 $[M-1]^+$	2 : 1 (5.5)	74 (20)	32
AND (intact) (290)	291.3 $[M+H]^+$	5 : 1 (6.1)	860 (250)	1
AND-NA (410)	411.2 $[M+H]^+$	3 : 2 (5.8)	5.2 (1.5)	170
AND-MNA (425)	425.3 $[M]^+$	2 : 1 (6.0)	2.1 (0.6)	410
Adiol (intact) (292)	257.2 $[M+H-2H_2O]^+$	3 : 1 (6.0)	750 (220)	1
Adiol-bisNA (532)	533.3 $[M+H]^+$	6 : 1 (6.3)	3.4 (1.0)	220
Adiol-bisMNA (562)	561.1 $[M-1]^+$	2 : 1 (5.0)	68 (20)	11

a) The proportions (v/v) of methanol–10 mM ammonium formate. *b*) The sensitivities of intact steroids are taken as 1. *c*) The values in parentheses are amounts (pg) converted into intact steroids.

oxosteroids could be easily detected at low femtomole levels, the latter showed about a two times higher sensitivity than the former except for DHEA. This result demonstrates that the charged derivatization is very useful for detecting trace amounts of steroids having only one utilizable functional group. For the di-oxosteroids, the HMP-derivatization was not quite effective in AD and resulted in an adverse effect in the sensitivity of PROG. In contrast, the bisHP derivatives of AD and PROG produced a satisfactorily high sensitivity; their LODs were as low as about 2 fmol. For hydroxysteroids, a similar phenomenon was observed. That is, quaternarization was effective for increasing the sensitivity for only the mono-hydroxysteroids (E_1 and AND), but the LODs of the bisMNA derivatives were much higher than those of the bisNA derivatives in E_2 and Adiol (about 20-fold). From these results, we can draw the conclusion that a pyridyl group is a readily ionizable moiety in the ESI process and its introduction is very valuable for increasing the detection responses of steroids having two of the same functional groups.

Advantage of HP- and NA-Derivatives in SRM Mode As previously reported, the HMP derivatives can provide a characteristic product ion derived from the HMP moiety at *m*/*z* 108 or 109, which were usable in the SRM mode.[11,12] Therefore, we examined the product ions of the bisHP and bisNA derivatives during MS–MS, because they will be employed as the monitoring ions in future biological sample analyses with the SRM mode.

The bisHP derivatives of AD and PROG provided a satisfactorily intense product ion at *m*/*z* 322 and 348, respectively. Although these ions could not be identified, it was inferred that they were formed by the loss of one HP moiety together with a part of the A-ring. Because the bisHP derivatives primarily have two easily ionizable HP moieties, the derivatives are still highly detectable in ESI-MS, even after the loss of one of them. Incidentally, the HP derivatives of mono-oxosteroids provided a product ion at *m*/*z* 95, which was $[\text{pyridine}+NH_2]^+$ formed by the cleavage of the N–N bond of the hydrazone.

In contrast to the bisHP derivatives, the bisNA derivatives of E_2 and Adiol gave the base product ion at *m*/*z* 139, which was assigned to the protonated pyridyl carbamic acid, and the

intensity of the product ion that originated from the steroid molecule was very low (Fig. 3b; the product ion mass spectrum of the bisNA derivative of Adiol is an example). The product ion at *m*/*z* 139 also had a satisfactory intensity and therefore, was expected to be usable for the SRM analysis.

Conclusion

The alternative derivatization procedure for charged derivatization was developed to enhance the detection responses of poorly ionizable steroids in ESI-MS. The HP- and NA-derivatization procedures were very useful for the analyses of di-oxosteroids and di-hydroxysteroids, respectively, for which the charged derivatization was unsuitable, and enable their detection in the low femtomole range. It is expected that these derivatization combined with LC–ESI-MS–MS can be applied for the trace level quantification of various steroids in biological fluids and tissues. Such studies are now in progress in our laboratories.

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