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2-Hydrazino-1-methylpyridine: a highly sensitive derivatization reagent for oxosteroids in liquid chromatography–electrospray ionization-mass spectrometry

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Abstract

A derivatization reagent, 2-hydrazino-1-methylpyridine, was developed for the liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) of oxosteroids. The reagent quantitatively reacted with oxosteroids at 60 °C within 1 h and the resulting derivatives of the mono-oxosteroids provided a 70–1600-fold higher sensitivity compared to intact steroids. However, HMP was unsuitable for di-oxosteroids, such as androstenedione and progesterone. The developed derivatization procedure was applied to the LC–ESI-MS analysis of 5α-dihydrotestosterone in human prostate, and allowed the reproducible quantification of nanogram/gram level of the androgen with a 10-mg sample.
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Keywords: Derivatization; LC–ESI-MS; Oxosteroids; Human prostate

1. Introduction

Many oxosteroids (Fig. 1) exert strong biological activities as steroid hormones (androgens and progestogens) at very low concentrations (nanomolar and even picomolar) in target tissues, and numerous synthetic analogues as well as natural oxosteroids have been used as therapeutic agents. A specific and sensitive method for the determination of these steroids in body fluids or tissues is necessary to elucidate the nature of many endocrine disease processes and thus be useful for diagnosis and treatment. Because of their close structural similarity, the metabolic versatility and their occurrence at low concentrations in body fluids and tissues, the development of reliable analytical methods of these steroids is a challenging subject for analytical chemists.

Numerous methods have been described for the characterization and determination of the steroids, such as immunoassay, receptor binding assay, high-performance liquid chromatography (HPLC) and gas chromatography (GC)–mass spectrometry (MS) [1,2]. Recently, liquid chromatography (LC) coupled with atmospheric pressure ionization, such as electrospray ionization (ESI)- and atmospheric pressure chemical ionization (APCI)-MS, has been used for these purposes due to its specificity and versatility. ESI-MS is widely used for the specific analysis of steroids [3,4], but apart from the glucuronidated and sulfated metabolites, the ionization efficiencies of most steroids are relatively low for ESI; conventional LC–ESI-MS sometimes does not demonstrate the required sensitivity for the trace analysis of steroids. To overcome this, chemical derivatization in LC–MS has recently been developed [5].

The best detectability with ESI-MS has been achieved in the analysis of compounds that are either ionic or can be readily ionized in solution. Based on this, the introduction of permanently charged moieties is effective for improving their sensitivities of neutral steroids in ESI-MS. Shackleton et al. [6] presented the LC–ESI-MS method of fatty acid

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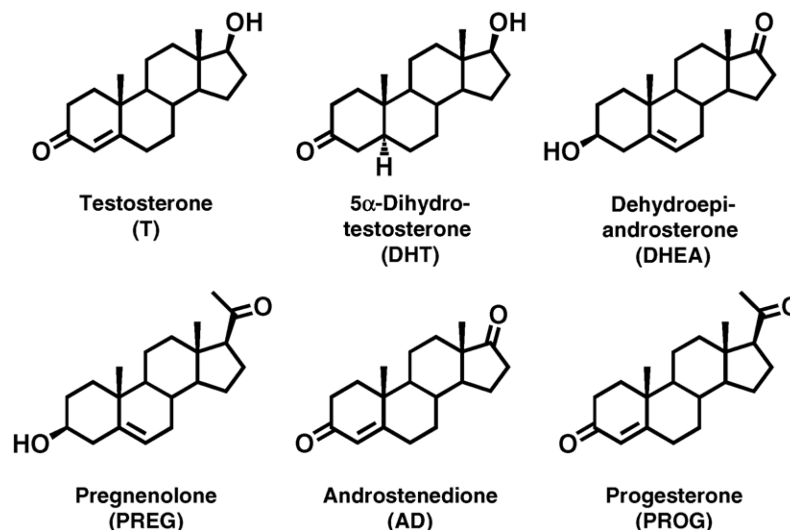

Fig. 1. Structures of oxosteroids examined in the present study.

esters of testosterone (T) following introduction of quaternary ammonium moiety, which increased the detectability of these non-polar steroids. One of the alternative useful charged moieties in the positive ESI-MS is the *N*-alkylpyridyl group (Fig. 2). Quirke et al. [7] demonstrated the utility of 2-fluoro-1-methylpyridine (FMP) as a derivatization reagent for alcohols. They reported that an intense molecular cation, $[M]^+$, was obtained in the positive ESI-MS when cholesterol was converted to its *N*-methylpyridyl ether. However, this procedure requires a large excess reagent with an aprotic solvent and the complicated purification step for removal of the reagent prior to the LC–MS analysis [8]. Girard reagent P (GP) is a derivatization reagent for carbonyl conpounds to form hydrazone derivatives with a quaternary pyridinium moiety and its application in LC–ESI-MS of oxosteroids has lately attracted considerable attention [9,10]. For example, Griffiths et al. [10] reported that the GP derivatives of some 3-oxosteroids, such as T, could be detected and identified on the sub-picograms level by ESI-MS. However, steroids with an oxo-group at the 17- or 20-position were less reactive than that at the 3-position, and therefore, the products of the reaction with GP were a mixture of the mono- and bis-hydrazones in the derivatization of androstenedione (AD) and progesterone (PROG). Thus, *N*-alkylpyridylation of steroids has a significant advantage for increasing the sensitivity in ESI-MS, but the derivatization reagents that are currently in use are not always satisfactory for practical applications. With this background information, we designed and synthesized 2-hydrazino-1-methylpyridine (HMP) as a new derivatization reagent with a quaternary pyridinium moiety for oxosteroids.

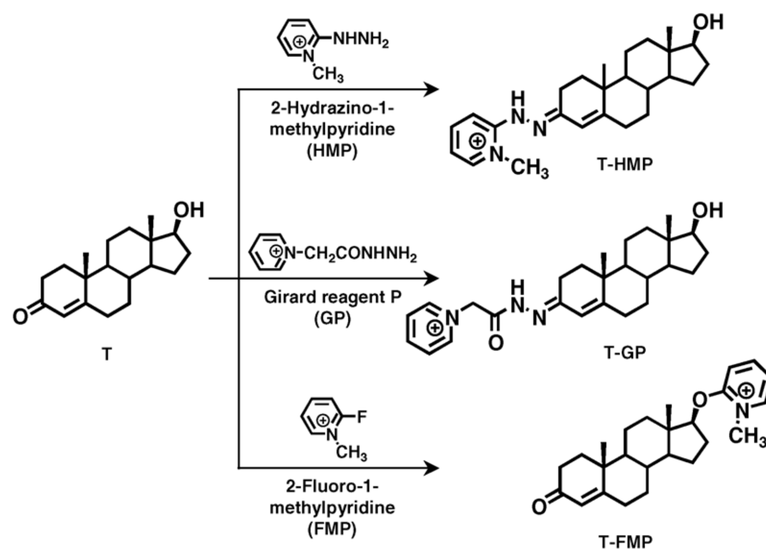

Fig. 2. Derivatization of T with HMP, GP and FMP.

In this paper, the property of HMP was examined. That is, HMP was compared with FMP and GP regarding the reactivity for oxosteroids and the chromatographic behavior and sensitivity of the resulting derivatives in LC–ESI-MS. The application of the developed derivatization method to biological sample analysis was also described.

2. Experimental

2.1. Materials and chemicals

T, 5α-dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), AD, pregnenolone (PREG) and PROG were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Stock solutions of each steroid were prepared as 50 μg/ml solutions in EtOH. Subsequent dilutions were carried out with EtOH to prepare 0.5, 1, 2, 5, 10 and 20 ng/ml solutions. (19,19,19-$^{2}H_3$)–T (D_3–T) [11] was donated by Teikoku Hormone Medical Research Center (Kawasaki, Japan). GP and FMP (*p*-toluenesulfonic acid salt) were obtained from Nacalai Tesque (Kyoto, Japan). 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. Bond Elut C18 cartridges (100 mg adsorbent; Varian, Harbor, CA, USA) were successively washed with MeOH containing 10% HCOOH (5 ml) and H_2O (5 ml) prior to use for the purification of the FMP derivatives. All other reagents and solvents were of analytical grade.

2.2. Preparation of HMP

To a solution of hydrazine hydrate (80%, 132 μl) in MeCN (30 ml), FMP (*p*-toluenesulfonic acid salt) (300 mg) in MeCN (6 ml) was added at 0 °C, and the mixture was stirred at 0 °C for 10 min and then at room temperature for 20 min under N_2. The resulting solution was concentrated and the residue was redissolved in MeCN (2 ml), which was filtered. After removal of the solvents from the filtrate, the crude product was recrystallized (twice) from MeCN–AcOEt (5:1, v/v) to give HMP (98 mg) as colorless needles (mp: 133–134 °C; ESI-MS: *m/z* 124.3 $[M]^+$). HMP was stored at 4 °C, at which it is stable for at least 6 months.

2.3. LC–MS(–MS)

LC–MS(–MS) was performed using an Applied Biosystems API 2000 triple stage quadrupole–mass spectrometer (Foster City, CA, USA) connected to a Shimadzu LC-10AT chromatograph (Kyoto). A J'sphere ODS H-80 column (4 μm, 150 mm × 2.0 mm i.d.; YMC, Kyoto) was used at a flow rate of 0.2 ml/min at 40 °C. Intact oxosteroids were analyzed by APCI-MS in the positive-ion mode (declustering potential: 30 V; focusing potential: 380 V; entrance potential: 10 V; nebulizer current: 2 μA; curtain gas: 45 psi; ion source gas 1: 80 psi; ion source gas 2: 15 psi; vaporizer temperature: 450 °C and interface heater: off). The HMP, FMP and GP derivatives of the oxosteroids were analyzed by ESI-MS in the positive-ion mode and the conditions were as follows: declustering potential: 80 V (HMP) or 30 V (FMP and GP); focusing potential: 200 V (HMP) or 380 V (FMP and GP); 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.

2.4. Derivatization of oxosteroids with HMP (optimized condition)

To a solution of the standard oxosteroid or pretreated biological sample in EtOH (30 μl), a freshly prepared solution of HMP (10 μg) in EtOH (50 μl) containing 25 μg of trifluoroacetic acid (TFA) was added, and the mixture was kept at 60 °C for 1 h. After removal of the solvents, the product was dissolved in the mobile phase, an aliquot of which was subjected to LC–MS.

2.5. Derivatization of oxosteroids with GP

To a solution of the standard oxosteroid in EtOH (30 μl), a freshly prepared solution of GP (10 μg) in EtOH (50 μl) containing 25 μg of TFA was added, and the mixture was kept at 60 °C for 1 h. After removal of the solvents, the product was dissolved in the mobile phase, an aliquot of which was subjected to LC–MS.

2.6. Derivatization of oxosteroids with FMP [8]

The standard oxosteroid was dissolved in CH_2Cl_2 (0.5 ml). To this solution, a freshly prepared solution of FMP (*p*-toluenesulfonic acid salt) (10 mg) in MeCN (150 μl) containing 6 μl of triethylamine was added, and the mixture was kept at room temperature for 1 h. After removal of the solvents, the residue was dissolved in H_2O (1 ml × 2) and applied to a Bond Elut C18 cartridge. After successive washing with H_2O containing 0.3% NH_3 (1 ml), H_2O (10 ml), MeOH (5 ml) and 50% MeOH containing 0.1% HCOOH (3 ml), the product was eluted with MeOH containing 10% HCOOH (5 ml). After evaporation, the product was dissolved in the mobile phase, an aliquot of which was subjected to LC–MS.

2.7. Optimization of reaction conditions in derivatization with HMP

Fifty nanograms of oxosteroids were derivatized with HMP (10 μg) in EtOH (50 μl) containing 25 μg of TFA at room temperature or 60 °C for 15, 30 or 60 min. The resulting derivatives were dissolved in MeOH–10 mM $HCOONH_4$ (1:1, v/v) (100 μl), 10 μl of which was subjected to HPLC (Shimadzu LC-6A chromatograph connected with Shimadzu SPD-10A UV detector (254 nm), column; J'sphere ODS H-80 (150 mm × 2.0 mm, i.d.) and flow rate; 0.2 ml/min). The

proportions of MeOH–10 mM HCOOH (v/v) used as the mobile phases of each compound were as follows: T–HMP and DHT–HMP; (2:1), DHEA–HMP; (3:2), PREG–HMP and AD–HMP; (5:2). The peak areas of the HMP derivatives formed under the respective reaction conditions were compared.

2.8. 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 compounds or derivatives per injection giving a signal to noise ratio (S/N) of 5). The base ions listed in Table 1 were monitored in the selected ion monitoring (SIM) mode. Oxosteroids (20 or 200 pg) were derivatized with HMP, GP or FMP as described above. These derivatives were dissolved in the mobile phases (100 μl) listed in Table 1 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 oxosteroids were determined using a 50% methanolic solution of 5, 10 or 100 ng/ml in the same way.

2.9. Pretreatment procedure for analysis of T and DHT in human prostate

Benign prostatic hyperplasia (BPH) tissue without any hormonal treatment was obtained from a patient, who gave informed consent at Gunma University (Maebashi, Japan), and stored at −20 °C prior to use. The tissue was minced by scissors and mashed by a glass homogenizer on ice. Ten milligrams of the mashed tissue was further homogenized in 30% MeOH (150 μl) by a glass homogenizer. The homogenate was heated at 60 °C for 30 min and centrifuged at 1500 × g (4 °C, 5 min). The supernatant was saved and the precipitate was suspended with 30% MeOH (150 μl) and heated at 60 °C for 30 min. After centrifugation at 1500 × g (4 °C, 5 min), the supernatants were combined (this solution is called the prostate extract). The prostate extract was added to MeCN (1 ml) containing D_3–T (200 pg), vortex-mixed for 30 s and centrifuged at 1500 × g (4 °C, 5 min). The supernatant was diluted with H_2O (3 ml) and purified using an Oasis HLB cartridge. After successive washing with H_2O (2 ml), 70% MeOH (2 ml) and hexane (1 ml), the steroids were eluted with AcOEt (1 ml). After evaporation, the residue was subjected to derivatization with HMP at 60 °C for 1 h. The resulting derivatives were dissolved in MeOH–10 mM $HCOONH_4$ (1:1, v/v) (30 μl), 10 μl of which was subjected to LC–MS.

2.10. Calibration curves for T and DHT

Each tube containing D_3–T (200 pg) and a mixture of T and DHT (each 5, 10, 20, 50, 100 or 200 pg) was derivatized with HMP at 60 °C for 1 h. The calibration curves were constructed by plotting the peak area ratios (T/D_3–T or DHT/D_3–T) against the amounts of T or DHT, respectively. Because the T and DHT derivatives gave twin peaks in the LC as described below, the respective main peaks (T–HMP; retention time (t_R) 7.8 min, DHT–HMP; t_R 6.9 min and D_3–T–HMP t_R 7.7 min) were used for the construction

Table 1
LODs of oxosteroids and their HMP, GP and FMP derivatives

Compound (mw)	Mobile phase[a]	t_R (min)	Base ion (m/z) (monitoring ion)	LOD (fmol)	Increasing sensitivity[b]
T (intact) (288)	2:1	7.0	289.2 $[M+H]^+$	69.4 (20 pg)[c]	1
T–HMP (394)	3:1	(5.9)[d], 7.6	394.1 $[M]^+$	1.0 (0.3 pg)	70
T–GP (422)	3:1	(6.4)[d], 7.6	422.3 $[M]^+$	10.4 (3.0 pg)	7
T–FMP (380)	3:5	6.3	380.2 $[M]^+$	5.2 (1.5 pg)	13
DHEA (intact) (288)	7:3	6.5	289.2 $[M+H]^+$	2780 (800 pg)	1
DHEA–HMP (394)	5:2	6.4	394.1 $[M]^+$	1.7 (0.5 pg)	1600
DHEA–GP (422)	1:1	6.5	422.3 $[M]^+$	4.2 (1.2 pg)	670
DHEA–FMP (380)	3:4	6.0	380.2 $[M]^+$	8.7 (2.5 pg)	320
PREG (intact) (316)	10:3	7.7	317.2 $[M+H]^+$	791 (250 pg)	1
PREG–HMP (422)	10:3	7.6	422.5 $[M]^+$	1.6 (0.5 pg)	500
PREG–GP (450)	4:3	7.1	450.2 $[M]^+$	1.6 (0.5 pg)	500
PREG–FMP (408)	1:1	6.3	408.2 $[M]^+$	63.3 (20 pg)	13
DHT (intact) (290)	2:1	7.2	291.2 $[M+H]^+$	276 (80 pg)	1
DHT–HMP (396)	3:1	(6.8)[d], 7.3	396.1 $[M]^+$	2.1 (0.6 pg)	130
AD (intact) (286)	2:1	6.0	287.2 $[M+H]^+$	52.4 (15 pg)	1
AD–bisHMP (498)	3:1	(5.8)[d], 7.7	497.1 $[M-1]^+$	52.4 (15 pg)	1
PROG (intact) (314)	3:1	6.8	315.2 $[M+H]^+$	15.9 (5.0 pg)	1
PROG–bisHMP (526)	6:1	(5.9)[d], 7.6	525.2 $[M-1]^+$	15.9 (5.0 pg)	1

[a] The proportions (v/v) of MeOH–H_2O (intact steroids), MeOH–10 mM $HCOONH_4$ (HMP and GP derivatives) and MeOH–H_2O containing 0.05% HCOOH (FMP derivatives).
[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 the calibration curves. MeCN–MeOH–10 mM $HCOONH_4$ (10:3:7, v/v/v) was used as the mobile phase.

3. Results and discussion

3.1. Optimization of reaction conditions in derivatization with HMP

The reactivity of HMP with the oxosteroids was examined by UV–HPLC. T, DHT, DHEA and RREG were used as model compounds for the 3-oxo-4-ene-steroids, saturated 3-oxosteroids, 17-oxosteroids and 20-oxosteroids, respectively. Due to the formation of the *E*- and *Z*-isomers during the derivatization of the oxosteroids with HMP, the resulting hydrazones usually have more than one peak in their chromatograms; the T and DHT derivatives had twin peaks, though we could not assign which of the two peaks was the *E*- or *Z*-isomer. On the contrary, the hydrazones of DHEA and PREG showed single peaks under the examined conditions. The t_{R}s of the respective derivatives under the LC conditions (Section 2.7) were as follows: T–HMP 12.5 and 17.3 min, DHT–HMP 14.6 and 16.0 min, DHEA–HMP 18.1 min and PREG–HMP 14.5 min.

HMP quantitatively reacted with T within 15 min at room temperature (Fig. 3); after the reaction for 15 min, intact T was not detected in the UV–HPLC chromatogram and the peak area of the HMP derivative (total of *E*- and *Z*-isomers) did not increase due to the longer reaction times or the heated condition. Griffiths et al. [10] have reported that GP quantitatively reacted with the 3-oxo-4-ene-steroids at 70 °C for 30 min when a large excess reagent (10 mg/sample) was used. In our supplementary experiment, such a large amount of reagent was not required for the preparation of the GP derivatives of the 3-oxo-4-ene-steroids on the ng–pg level (see Section 2.5). However, the heating was necessary for the

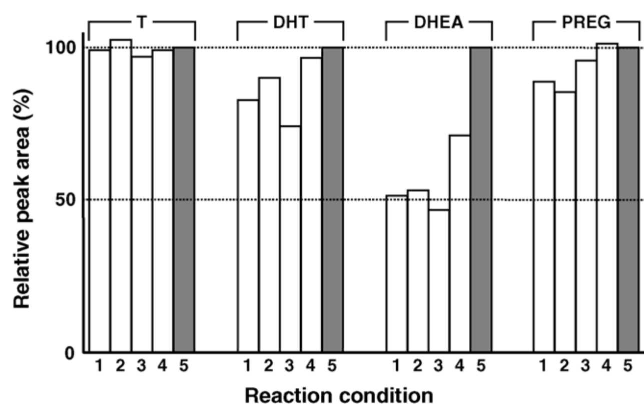

Fig. 3. Reactivity of HMP for oxosteroids. Reaction conditions; (1): room temperature, 15 min, (2): room temperature, 30 min, (3): 60 °C, 15 min, (4): 60 °C, 30 min and (5): 60 °C, 60 min. The peak areas of the derivatives formed under condition 5 were taken as 100%. In the T and DHT derivatives, total peak areas of the isomers were used.

quantitative derivatization of the 3-oxo-4-ene-steroids with GP.

Although over 85% of PREG reacted with HMP at room temperature for 30 min, heating was required to increase the degree of the derivatization. By heating (60 °C), the derivatization in PREG rapidly proceeded and was completed within 30 min. The derivatization of PREG with GP was reported to be required for the longer reaction times (more than 30 min) with heating (70 °C) [10]. Based on these results, HMP is more reactive with oxosteroids than GP. One of the reasons for this is that the reacting group of HMP is hydrazine, that is more reactive with an oxo-group than the hydrazide of GP.

Because the 17-oxo-group was less reactive than the 20-oxo-group, the longer reaction times with heating were required to complete the derivatization in DHEA as shown in Fig. 3. We considered that the derivatization of DHEA with HMP was completed at 60 °C within 1 h, based on the following result. For the derivatization of AD, that contains both the 3-oxo-4-ene-structure and 17-oxo-group, the reaction product was a mixture of the 3-mono- and 3,17-bis-hydrazones at the ratio of ca 5:2 for 30 min of reaction time, but bis-hydrazone was the only reaction product after a 1 h reaction time.

A saturated 3-oxosteroid, DHT, was less reactive than a 3-oxo-4-ene-steroid, T, in the reaction with HMP. However, the reaction seemed to be completed within 30 min with heating, because the yield of the derivative did not significantly increase with the longer reaction time.

Based on the above results, it is concluded that the HMP derivatives of the 3-, 17- and 20-oxosteroids are quantitatively formed under the condition described in Section 2.4 (60 °C, 1 h); T, DHT, DHEA and PREG were tagged with one HMP group and AD and PROG were tagged with two HMP groups.

3.2. ESI-MS(–MS) of HMP derivatives of oxosteroids

For the ESI-MS operating in the positive-ion mode, all the HMP derivatives of the mono-oxosteroids (T, DHEA, PROG and DHT) provided only their molecular cations, $[M]^+$ (Fig. 4a and Table 1). On the contrary, the derivatives of AD and PROG gave $[M-1]^+$ together with some characteristic fragment ions. We cannot sufficiently explain why these bis-HMP derivatives gave $[M-1]^+$, but not $[M]^+$. In AD-bis HMP, $[M-1]^+$ at *m/z* 497.3 was the base ion and fragment ions, $[M-1-108]^+$ (loss of (*N*-methylpyridine + NH)), $[M-1-122]^+$ (loss of HMP), $[M-162]^+$ (not identified) and $[N\text{-methylpyridine}+\mathrm{NH}]^+$, were observed at *m/z* 389.3, 375.3, 336.3 and 107.9, respectively. PROG-bisHMP also gave $[M-1-122]^+$ (loss of HMP), $[M-164]^+$ (not identified) and $[N\text{-methylpyridine}+\mathrm{NH}]^+$ at *m/z* 403.4, 362.1 and 107.9, respectively, as the fragment ions. $[M]^{2+}$ ions were also detected in these bis-HMP derivatives, but their intensities were below 30% of the respective $[M-1]^+$ ions.

The MS–MS analysis of the HMP derivative was also evaluated using T–HMP as the model compounds, where $[M]^+$ was used as the precursor ion (Fig. 4b). Griffiths et al. have

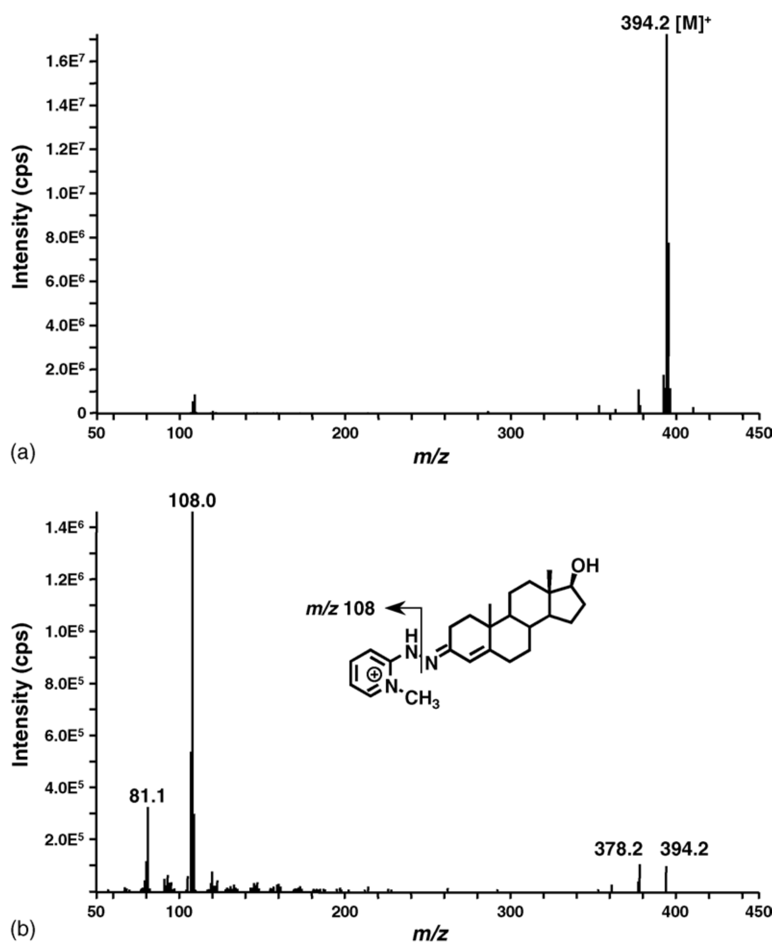

Fig. 4. (a) ESI-MS and (b) ESI-MS–MS spectra of T–HMP. Ten nanograms of the derivative was subjected to LC–MS.

shown that the GP derivatives of the oxosteroids give many structurally informative product ions by the low- [10] and high-energy collision-induced dissociation [12]. However, $[M]^+$ of the HMP derivative was so stable that no product ion was formed by the addition of less than a 30 eV collision energy. When a 60 eV collision energy was given, only two product ions were observed at *m/z* 81.1 and 108.0. The former product ion could not be identified and the latter one was assigned as $[N\text{-methylpyridine} + NH]^+$ formed by the cleavage of the N–N bond of the hydrazone. Based on these results, the following two selected reaction monitoring (SRM) methods were considered to be applicable to the analysis of the HMP derivative. One method employed a 60 eV collision energy and monitored the product ion at *m/z* 108.0, though its intensity was significantly weak in comparison with that of the precursor ion (about 10%). In the other method, the residual $[M]^+$ was monitored after the collision with a 30 eV energy. By this technique, it was expected that the noise ions were reduced without decreasing the intensity of the monitoring ion. Compared these two MS–MS modes with emphasis on the *S/N* value, there was no significant difference between two modes. Furthermore, the LODs in the SIM and SRM modes were almost same in the standard sample analysis. Based on this, the SIM mode of the monitoring $[M]^+$ of the respective steroids was used for the evaluation of the effect of the derivatization (Section 3.4).

We also compared the sensitivity between the SIM and SRM modes in the GP derivatives. The derivatives provided intense $[M]^+$ in ESI-MS and several product ions by MS–MS (collision energy: 40 eV). The most abundant product ion was $[M-79]^+$, which was formed by the elimination of the pyridine moiety from $[M]^+$ [10], but its intensity was never intense (about 5% of the intensity of $[M]^+$). The LODs in the SRM mode (precursor ions: $[M]^+$ and monitoring ions $[M-79]^+$) were almost equal or inferior to those in the SIM mode in the GP derivatives. Thus, the SRM mode was not an advantage in the standard sample analysis of the GP derivatives as well as the HMP derivatives, though this mode may demonstrate its ability in the biological sample analysis.

3.3. Comparison of HMP derivatives with GP and FMP derivatives in chromatographic behavior

As mentioned above, due to the formation of the *E*- and *Z*-isomers during the derivatization, the HMP derivatives of steroids having a 3-oxo-group (T, DHT, AD and PROG) had

twin peaks. On the contrary, the derivatives of DHEA and PREG, steroids having only a 17- or 20-oxo-group, showed single peaks (Table 1).

The chromatographic behavior of the HMP derivatives was compared with that of the GP derivatives. Both derivatives had twin peaks corresponding to the *E*- and *Z*-isomers. The GP derivatives showed a significant peak tailing, which is a major concern for the separation of the measuring steroids from endogenous interfering substances in biological samples, thus leading to a low assay accuracy and precision. The alternations of the LC condition, that is, change of the organic modifier (from MeOH to MeCN), increase of the $HCOONH_4$ concentration (20 mM), pH adjustment (pH 5.5 and 7.5) and use of the different analytical columns (Chemcobond ODS-W (150 mm × 2.1 mm i.d.; Chemoco Scientific, Osaka, Japan), Develosil ODS-HG-5 (150 mm × 2.0 mm i.d.; Nomura Chemical, Seto, Japan) and Capcell Pak C18 type MG (150 mm × 2.0 mm i.d.; Shiseido Fine Chemicals, Tokyo)), were not effective for improving the peak tailing in the GP derivatives. On the other hand, the HMP derivative gave satisfactory shaped peaks. The chromatograms of T–HMP and T–GP are shown in Fig. 5a and b, respectively, as examples. These data demonstrate that HMP is superior to GP in the chromatographic behavior of the resulting derivatives. The FMP derivatives gave single peaks with satisfactory shapes (Fig. 5c), when the mixture of MeOH and 0.05% HCOOH [8] were used as the mobile phases.

3.4. Effect of derivatization with HMP for the detection responses in oxosteroids analyses

The effects of the respective derivatizations for the detection responses were evaluated by the LODs, where the mobile phases were adjusted so that the t_R values of the respective steroid derivatives were between 6 and 8 min (when a derivative gave twin peaks, its major peak was used for this study) (Table 1). Because intact oxosteroids provided their protonated molecular ions ($[M+H]^+$) more efficiently in APCI than ESI, their LODs were determined in the APCI mode.

Although the GP-derivatization provided intense molecular cations and provided the pg-level detection of steroids as described by Griffiths et al. [10], the HMP derivatives of T, DHEA and PREG showed a higher or equivalent sensitivity. The effect of HMP for DHEA was particularly impressive; the HMP-derivatization increased the sensitivity by 1600-fold over the intact DHEA. In DHT, the HMP-derivatization also had a significantly increased sensitivity. The FMP derivatives of T, DHEA and PREG also gave intense $[M]^+$ and their LODs were much better than those of the intact steroids, but inferior to those of the HMP derivatives, though FMP and HMP contain the same charged moiety, *N*-methylpyridine. One of the reasons for this is considered to be the loss of the FMP derivatives during the purification step after the derivatization. That is, as mentioned in the introductory section, the FMP-derivatization requires a large excess reagent due to the low reactivity of the reagent, and because of this, the complicated purification steps for removal of the reagent prior to the LC–MS analysis were required. Thus, HMP has the greater validity in increasing the sensitivity of mono-oxosteroids in ESI-MS than the reagents that are currently in use, i.e., GP and FMP.

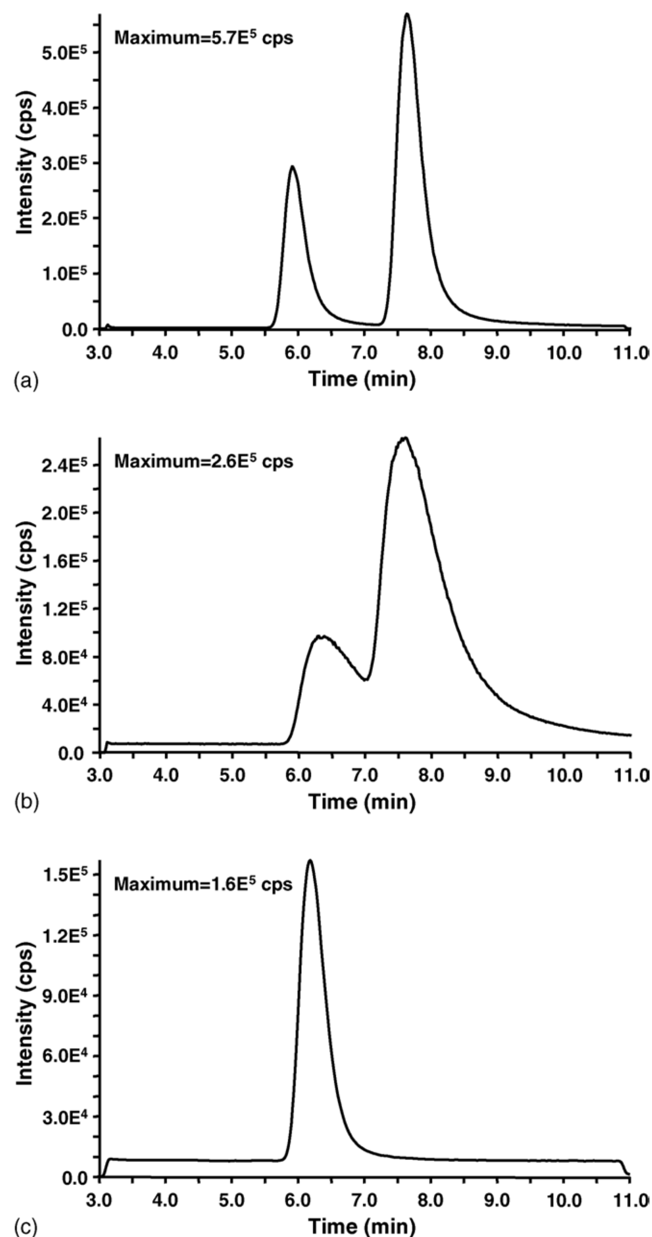

Fig. 5. Chromatograms of (a) T–HMP, (b) T–GP and (c) T–FMP. One hundred nanograms of T was derivatized and then dissolved in the mobile phases (100 μl). These derivatives (1 μl) were analyzed by the SIM mode. Mobile phases and monitoring ions are listed in Table 1.

However, for AD and PROG, the LODs of their HMP derivatives were little better than those of the intact steroids. For this result, the following two subjects are considered to be the major factors. The first is that the bis-HMP derivatives provide a multiple number of ions, $[M-1]^+$, $[M]^{2+}$ and fragment ions, during their ionization process as described

above. The second is that the desolvation of the liquid-phase ions in a droplet, in other words, the formation of gas-phase ions from liquid-phase ions, may inefficiently proceed due to the highly hydrophilic properties of the bis-HMP derivatives. Incidentally, we are now examining whether the similar phenomenon is observed for the bis-FMP derivatives of the dihydroxysteroids.

3.5. Application of HMP-derivatization to prostatic androgen analysis

To investigate the possibility of the proposed derivatization with HMP for biological sample analysis, the primary quantitative study of androgens in human prostate was performed. The most active androgen, DHT and its precursor, T, were chosen as the target analytes, because of our interest in their potential roles in prostatic diseases, such as BPH and prostate cancer, which are sharply increasing in Japan.

The prostate extract prepared from a 10-mg tissue of a patient with BPH was pretreated, derivatized with HMP and analyzed by LC–MS. To obtain a greater sensitivity and specificity, the MS–MS mode with a 30 eV collision energy using $[M]^+$ ions as the precursor ions and the same residual ions as the monitoring ions was employed in the prostate analysis. D_3–T was used as the internal standard. Because the difference in *m/z* of the $[M]^+$ ions of T–HMP and DHT–HMP is only 2 units and the isotopic molecular ions of T–HMP have some influence on the peak area of DHT–HMP, the chromatographic separation of these derivatives are required for their accurate quantification. For a similar reason, the DHT and D_3–T derivatives (*m/z* 396.1 and 397.1, respectively) should be separated during LC. Because these derivatives

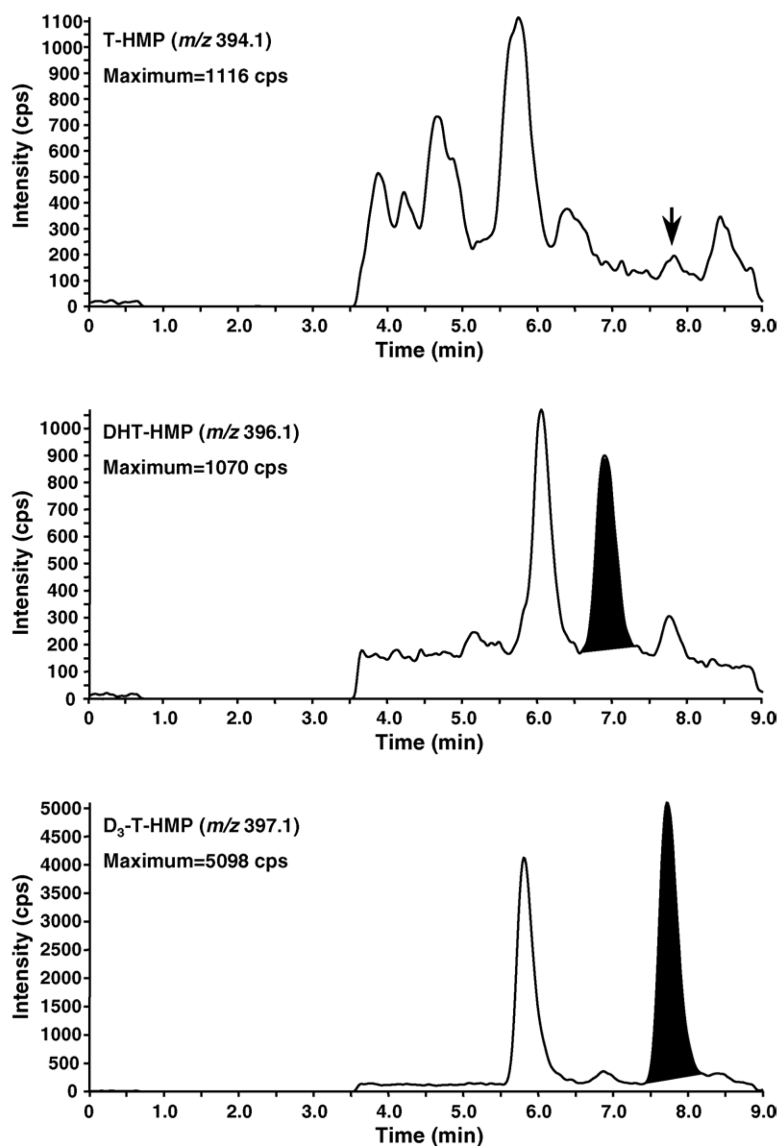

Fig. 6. Chromatograms of derivatized T, DHT and D_3–T in human prostate. Mobile phase: MeCN–MeOH–10 mM $HCOONH_4$ (10:3:7, v/v/v). The arrow indicates the elution position of the T derivative. The measured concentration of T and DHT in the prostate were less than 0.5 and 5.66 ng/g tissue, respectively.

consisted of isomers as mentioned above, it was very hard to completely separate all the peaks derived from the isomers. However, when MeCN–MeOH–10 mM $HCOONH_4$ (10:3:7, v/v/v) was used as the mobile phase, the satisfactory chromatographic and mass separation of the major isomers of the derivatives was achieved; T–HMP m/z 394.1 and t_R 7.8 min. DHT–HMP m/z 396.1 and t_R 6.9 min and D_3–T–HMP m/z 397.1 and t_R 7.7 min. Therefore, the peaks derived from the major isomers were used for the determination of T and DHT. The regression lines for T and DHT showed a good linearity in the range of 5–200 pg/tube; y (T/D_3–T) = 0.00388x + 0.0091, with a correlation coefficient (r^2) of 0.999 and y (DHT/D_3–T) = 0.00328x + 0.0149 with r^2 of 0.997. Comparison with the t_R values of the reference compounds indicated that the peak at 6.9 min in the SRM chromatogram of m/z 396.1 of the prostate sample corresponded to the DHT derivative (Fig. 6). The level of T was below the detection limit in this sample (the arrow indicates the elution position of the major isomer of the T derivative). The level of DHT in the prostate presented in Fig. 6 was determined to be 5.41 ± 0.44 ng/g tissue (mean ± standard deviation (S.D.) ($n = 5$)) with an intra-assay coefficient of variation (CV) of 8.1% for DHT. The inter-assay CV ($n = 5$) was 9.3% (measured value: 5.66 ± 0.53 ng/g tissue). These results demonstrate that the present method enables the precise quantification of small amounts of DHT in the prostate with a sample collected for the microscopic diagnosis using a biopsy needle (ca. 10-mg tissue).

4. Conclusion

The new derivatization reagent, HMP, was developed for the LC–ESI-MS analysis of oxosteroids. This reagent was superior to the reagents that are currently in use based on the reactivity, handling after derivatization, and chromatographic behavior and sensitivity of the resulting derivatives in LC–ESI-MS of mono-oxosteroids. However, HMP was unsuitable for di-oxosteroids, such as androstenedione and progesterone. The HMP-derivatization enabled the detection of nanogram/gram level of DHT in the human prostate with a 10-mg tissue sample. The primary quantitative study also revealed that the LC–MS method using this derivatization was reproducible. More rigorous analyses for the elucidation of the roles of androgens in prostate diseases can be performed when further validation studies have been carried out. It is also expected that this derivatization combined with LC–ESI-MS can be applied for the trace level quantification of various oxosteroids in biological fluids and tissues, such as neurosteroids in the brain [13], as well as prostate androgens. Such studies are now in progress in our laboratories.

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