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Electron-capturing Derivatization of Neutral Steroids for Increasing Sensitivity in Liquid Chromatography/Negative Atmospheric Pressure Chemical Ionization–Mass Spectrometry

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Derivatization of neutral steroids for increasing sensitivity in liquid chromatography/negative atmospheric pressure chemical ionization–mass spectrometry (APCI-MS) has been examined. Under APCI conditions, gas-phase electrons are provided by the corona discharge and captured by electron-affinitive compounds. In negative APCI-MS, therefore, ultrahigh sensitivity can be obtained by tagging neutral steroids, whose ionization efficiencies are low in the conventional APCI-MS, with electron-capturing moieties, such as a nitro group. We synthesized various boronic acid and hydrazine derivatives having electron-capturing moieties as derivatization reagents for 1,2-diol compounds and oxosteroids, respectively. Among reagents examined, those having the 2-nitro-4-trifluoromethylphenyl moiety were most effective in increasing sensitivity. That is, the detection responses of the derivatives with these reagents were increased by several to more than 200-fold over intact steroids, where limits of detection were some picograms. The developed derivatization procedures were applied to analyses of small amounts of steroids in human plasma and gave satisfactory results.

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Introduction

Steroid hormones, including vitamin D metabolites (9,10-secosteroids), exert strong biological activities at very low concentrations in target tissues. A specific and sensitive method for the determination of the 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. Recently liquid chromatography (LC) together with atmospheric pressure ionization (API)-based mass spectrometry (MS) has been used for this purpose due to its specificity and versatility.[1] However, except for conjugated steroids, because most steroids have a few polar functional groups, their ionization efficiencies are relatively low for various ionization methods. Therefore, conventional LC/MS sometimes does not demonstrate the required sensitivity for trace analysis of steroids. To overcome this, derivatization suitable for the each ionization method has been examined.

Because electrospray ionization (ESI)-MS is useful for the analysis of permanently charged compounds, some neutral steroids are analyzed using this technique after conversion to the charged forms, such as a hydrazone having a quaternary pyridinium moiety[2] and a protonated oxime[3] for positive-ion detection, and a sulfate[4] and a carboxymethyloxime[5] for negative-ion detection. In ESI-MS, however, analyte signals are relatively sensitive to suppression by contaminants from the biological matrix.[6] Atmospheric pressure chemical ionization (APCI) is also widely used as an API technique, especially for neutral compounds, in which ion suppression by contaminants less frequently occurs compared to ESI. In positive APCI-MS, the introduction of proton-affinitive atoms, such as oxygen and nitrogen, to the analyte is very effective in increasing sensitivity of the resulting derivative. Based on this information, we have developed the LC/positive APCI-MS methods for the determination of vitamin D compounds[7,8] and neurosteroids[9] after conversion to their Diels–Alder adducts with Cookson-type reagents and methyloximes, respectively. On the other hand, APCI operating in the negative-ion mode is expected to provide greater sensitivity, because background noise is relatively low in this mode.

In APCI, the corona discharge can provide a source of electrons in the gas-phase. This can be an advantage for some compounds that can undergo electron capture. Singh *et al.*[6] reported that very high sensitivity was obtained for the LC/negative APCI-MS analysis of pentafluorobenzyl (PFB) derivatives of estrogens, where the derivatives generated negative ions through the loss of the PFB group ($[M–PFB]^-$), but not deprotonated molecular ions ($[M–H]^-$). Singh *et al.* consequently termed this technique electron capture APCI (ECAPCI). Thus, usefulness of introduction of the PFB group in negative APCI-MS has been demonstrated, but effects of other functional groups, such as a nitro group, which is a representative electron-affinitive group, have been poorly studied.

In the present paper, we first synthesized various boronic acid derivatives having some nitro and trifluoromethyl groups, which were designed for analysis of 1,2-diol compounds. Then, their effects in increasing sensitivity in negative APCI-MS were examined using 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] as a model compound and the most effective reagent was applied to the analysis of 24,25$(OH)_2D_3$ in human plasma. Hydrazine derivatives for oxosteroids were also synthesized, one of which was employed for the simultaneous analysis of testosterone (T), dehydroepiandrosterone (DHEA) and pregnenolone (PREG) in human plasma.

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Table 1 LC/MS conditions and LODs of 24,25$(OH)_2D_3$ and its boronates

Derivative	Mobile phase (t_R/min)	APCI-MS conditions[a]			Monitoring ion	LOD (S/N = 3)	Increasing sensitivity
Intact	MeOH–H_2O, 9:2 (6.7)	475˚C	175˚C	80 units	Sum of 417.0 $[M + H]^+$, 399.0 $[417–H_2O]^+$ and 381.0 $[417–2H_2O]^+$	216 fmol (90 pg)	1
NP-APB	MeCN–H_2O, 97:3 (6.4)	375˚C	250˚C	60 units	638.3 $[M]^-$	4.8 fmol	45
DNP-APB	MeCN–H_2O, 49:1 (6.2)	375˚C	225˚C	80 units	683.3 $[M]^-$	1.7 fmol	129
TNP-APB	MeCN–H_2O, 23:2 (6.6)	425˚C	225˚C	60 units	728.3 $[M]^-$	3.6 fmol	60
NBD-APB	MeCN–H_2O, 47:3 (6.2)	475˚C	225˚C	60 units	680.3 $[M]^-$	3.8 fmol	56
2NFP-APB	MeCN–MeOH, 93:7 (6.9)	325˚C	225˚C	60 units	706.3 $[M]^-$	0.96 fmol	225
4NFP-APB	MeCN–H_2O, 99:1 (6.7)	425˚C	225˚C	60 units	706.3 $[M]^-$	6.0 fmol	36
DNFP-APB	MeCN–H_2O, 47:3 (6.5)	375˚C	225˚C	60 units	751.1 $[M]^-$	1.9 fmol	112
DNPy-APB	MeCN–H_2O, 24:1 (6.1)	425˚C	225˚C	60 units	684.3 $[M]^-$	1.9 fmol	112
PFB-APB	MeCN–H_2O, 99:1 (6.3)	325˚C	175˚C	80 units	709.1[b]	6.7 fmol	32

a. Vaporizer temperature, capillary temperature and sheath gas flow rate, respectively. b. The assignment of the ion has not been done.

Table 2 LC/MS conditions and LODs of PREG and its hydrazones

Derivative	Mobile phase (t_R/min)	APCI-MS conditions[a]			Monitoring ion	LOD (S/N = 3)	Increasing sensitivity
Intact	MeOH–H_2O, 4:1 (6.0)	550˚C	200˚C	60 units	299.0 $[M+H–H_2O]^+$	269 fmol (85 pg)	1
DNPH	MeCN–H_2O, 8:1 (6.5)	300˚C	225˚C	60 units	496.2 $[M]^-$	47 fmol	6
2NFPH	MeCN–H_2O, 30:1 (6.4)	275˚C	225˚C	60 units	519.2 $[M]^-$	16 fmol	17
DNFPH	MeCN–H_2O, 7:1 (6.2)	375˚C	225˚C	60 units	563.9 $[M]^-$	57 fmol	5

a. Vaporizer temperature, capillary temperature and sheath gas flow rate, respectively.

Experimental

Material and reagents

24,25$(OH)_2D_3$ was obtained from Duphar B. V. Co. (Amsterdam, The Netherlands). Oxosteroids (T, DHEA and PREG) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). [26,26,26,27,27,27-$^2H_6$]-24,25$(OH)_2D_3$ {[$^2H_6$]-24,25$(OH)_2D_3$} was prepared in our laboratories.[7] 2,4-Dinitrophenylhydrazine (DNPH) was obtained from Kanto Chemical (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. Bond Elut Si cartridges (500 mg adsorbent; Varian, Harbor, CA, USA) were successively washed with AcOEt (4 mL) and hexane (4 mL) prior to use. All other reagents and solvents were of analytical grade.

LC/MS(/MS)

LC/MS(/MS) was performed using a ThermoQuest LCQ (San Jose, CA) liquid chromatograph-ion trap-mass spectrometer connected to a JASCO PU-980 (Tokyo) chromatograph, and APCI was employed. A J'sphere ODS H-80 (4 μm, 150 × 4.6 mm i.d.; YMC, Kyoto, Japan) column was used at a flow rate of 1 mL min^{-1} at 40˚C. For the MS/MS analysis, helium was used as the collision gas. Intact 24,25$(OH)_2D_3$ (capillary voltage and tube lens offset voltage; 3 V and 15 V, respectively) and oxosteroids (10 V and 15 V) were analyzed in the positive-ion mode and boronates of 24,25$(OH)_2D_3$ (–3 V and –15 V) and hydrazones of oxosteroids (–10 V and –15 V) were analyzed in the negative-ion mode. The other MS conditions and mobile phases for each analyte are described in Tables 1, 2 or Table 3.

In the analysis of 24,25$(OH)_2D_3$ and oxosteroids in human plasma, ZoomScan mode, a higher resolution technique in the ThermoQuest LCQ mass spectrometer, was used.

Syntheses of boronic acid derivatives

General: Silica-gel column chromatography was carried out with a Merck silica-gel 60 (60 – 200 μm; Darmstadt, Germany). Structural confirmation of boronic acid derivatives was performed by flow injection/negative ESI-MS [MeOH–H_2O, 1:1 (v/v), flow rate 0.5 mL min^{-1}, sheath gas flow rate 80 units, auxiliary gas flow rate 20 units, capillary temperature 200˚C, capillary voltage –5 V, and tube lens offset voltage –15 V]. Crystallization of the synthesized samples failed in spite of our efforts, so that melting points were not measured. The part of the sample {one spot in thin layer chromatography [TLC; Merck silica-gel $60F_{254}$ pre-coated plate, $CHCl_3$–MeOH, 20:1 (v/v)]} was used for the derivatization and the exact yields of each sample from 3-aminophenyldihydroxyborane (APB; starting material) were not measured.

[3-(4-Nitrophenyl)aminophenyl]dihydroxyborane (NP-APB): 4-Fluoronitrobenzene (0.3 mL) was added to a solution of APB (50 mg) in dimethyl sulfoxide (DMSO)–1 M Na_2CO_3, 1:1 (v/v) (2 mL), and the mixture was kept at 70˚C for 6 h with occasional vortex-mixing. The resulting solution was diluted with AcOEt and washed with H_2O, and then dried over anhydrous Na_2SO_4. After removal of solvents, the crude product was purified by silica-gel column chromatography (25 × 1 cm i.d.) [$CHCl_3$–MeOH, 20:1 (v/v)] to give NP-APB as a yellow solid. ESI-MS; m/z 257.3 $[M–H]^-$ (100%).

[3-(2,4-Dinitrophenyl)aminophenyl]dihydroxyborane (DNP-APB): 2,4-Dinitrofluorobenzene (0.3 mL) was added to a solution of APB (50 mg) in DMSO–1 M Na_2CO_3, 1:1 (v/v) (2 mL), and the mixture was kept at 60˚C for 1 h. The mixture was treated in the same way as NP-APB to give DNP-APB as an orange solid. ESI-MS; m/z 302.2 $[M–H]^-$ (100%).

[3-(2,4,6-Trinitrophenyl)aminophenyl]dihydroxyborane (TNP-APB): 2,4,6-Trinitrobenzenesulfonic acid sodium salt dihydrate (200 mg) was added to a solution of APB (50 mg) in 1 M Na_2CO_3 (1 mL), and the mixture was kept at 60˚C for 30 min. The mixture was treated in the same way as NP-APB to

Table 3 LODs of oxosteroids and their 2NFPH derivatives

	T	DHEA	PREG
Intact compounds (positive-ion mode)			
Mobile phase	MeOH–H_2O, 2:1	MeOH–H_2O, 7:3	MeOH–H_2O, 4:1
t_R (min)	6.9	6.3	6.0
Monitoring ion	289.0 $[M + H]^+$	271.0 $[M+H–H_2O]^+$	299.0 $[M+H–H_2O]^+$
LOD (S/N = 3, fmol)	69 (20 pg)	382 (110 pg)	269 (85 pg)
2NFPH derivatives (negative-ion mode)			
Mobile phase	MeCN–H_2O, 15:1	MeCN–H_2O, 11:1	MeCN–H_2O, 30:1
t_R (min)	6.4, 6.7[a]	6.5	6.4
Monitoring ion	491.2 $[M]^-$	491.2 $[M]^-$	519.2 $[M]^-$
LOD (S/N = 3, fmol)	10[b]	10	16
Increasing sensitivity	7	37	17

a. Two peaks for *syn* and *anti* isomers; 6.4 (minor) and 6.7 (major). b. Major peak (6.7 min) was used for the determination of LOD.

give TNP-APB as a brown solid. ESI-MS; *m*/*z* 347.2 $[M–H]^-$ (100%).

4-[*N*-(3-Dihydroxyboryl)phenyl]amino-7-nitro-2,1,3-benzoxadiazole (NBD-APB): 4-Chloro-7-nitro-2,1,3-benzoxadiazole (90 mg) was added to a solution of APB (50 mg) in MeCN–1 M Na_2CO_3, 3:4 (v/v) (21 mL), and the mixture was stirred at 60˚C for 1 h. The mixture was treated in the same way as NP-APB to give NBD-APB as a brown solid. ESI-MS; *m*/*z* 299.2 $[M–H]^-$ (100%).

[3-(2-Nitro-4-trifluoromethylphenyl)aminophenyl]dihydroxyborane (2NFP-APB): 4-Fluoro-3-nitrobenzotrifluoride (0.3 mL) was added to a solution of APB (50 mg) in DMSO–1 M Na_2CO_3, 1:1 (v/v) (2 mL), and the mixture was kept at 60˚C for 1 h. The mixture was treated in the same way as NP-APB to give 2NFP-APB as a yellow solid. ESI-MS; *m*/*z* 325.2 $[M–H]^-$ (100%).

[3-(4-Nitro-2-trifluoromethylphenyl)aminophenyl]dihydroxyborane (4NFP-APB): 2-Fluoro-5-nitrobenzotrifluoride (0.3 mL) was added to a solution of APB (50 mg) in DMSO–1 M Na_2CO_3, 1:2 (v/v) (1.5 mL), and the mixture was kept at 60˚C for 5 h with occasional vortex-mixing. The mixture was treated in the same way as NP-APB to give 4NFP-APB as a yellow solid. ESI-MS; *m*/*z* 325.2 $[M–H]^-$ (100%).

[3-(2,4-Dinitro-6-trifluoromethylphenyl)aminophenyl]dihydroxyborane (DNFP-APB): 2-Chloro-3,5-dinitrobenzotrifluoride (200 mg) was added to a solution of APB (50 mg) in DMSO–1 M Na_2CO_3, 1:2 (v/v) (1.5 mL), and the mixture was kept at 60˚C for 1 h. The mixture was treated in the same way as NP-APB to give DNFP-APB as an orange solid. ESI-MS; *m*/*z* 370.2 $[M–H]^-$ (100%).

[3-(2,4-Dinitropyridyl)aminophenyl]dihydroxyborane(DNPy-APB): 2-Chloro-3,5-dinitropyridine (131 mg) was added to a solution of APB (50 mg) in DMSO–1 M Na_2CO_3, 1:2 (v/v) (1.5 mL), and the mixture was kept at 60˚C for 10 min. The mixture was treated in the same way as NP-APB. The brown solid from silica-gel column chromatography was reprecipitated from $CHCl_3$–MeOH to give DNPy-APB as an orange solid. ESI-MS; *m*/*z* 303.2 $[M–H]^-$ (100%).

[3-(2,3,4,5,6-Pentafluorobenzyl)aminophenyl]dihydroxyborane (PFB-APB): To a suspension of APB (50 mg) in MeCN (0.3 mL), 2,3,4,5,6-pentafluorobenzyl bromide (60 µl) was added, and the mixture was kept at room temperature for 1 h. The resulting solution was concentrated, diluted with AcOEt and washed with H_2O, and then dried over anhydrous Na_2SO_4. After removal of solvents, the crude product was purified by column chromatography (25 × 1 cm i.d.) [AcOEt–hexane, 4:3 (v/v)] to give PFB-APB as a colorless solid. ESI-MS; *m*/*z* 316.2 $[M–H]^-$ (30%), 348.1 $[M–H+O_2]^-$ (100%).

Syntheses of hydrazine derivatives

General: The structure and purity of the synthesized hydrazine derivatives were confirmed using flow injection/negative ESI-MS and TLC, respectively, in a similar manner as done for the boronic acid derivatives. Melting points were measured on a Yanaco MP-J3 (Kyoto) melting point apparatus without correction.

2-Nitro-4-trifluoromethylphenylhydrazine (2NFPH): To a solution of 4-fluoro-3-nitrobenzotrifluoride (100 µL) in MeCN (1 mL), hydrazine hydrate (80%, 110 µL) was added, and the mixture was kept at room temperature for 15 min. The resulting solution was concentrated, diluted with AcOEt and washed with H_2O, and then dried over anhydrous Na_2SO_4. After removal of solvents, the crude product was recrystallized from MeOH (twice) to give 2NFPH as orange needles (mp 116 – 116.5˚C). ESI-MS; *m*/*z* 220.0 $[M–H]^-$ (100%).

2,4-Dinitro-6-trifluoromethylphenylhydrazine (DNFPH): To a solution of 2-chloro-3,5-dinitrobenzotrifluoride (100 mg) in MeCN (1 mL), hydrazine hydrate (80%, 44 µL) was added, and the mixture was kept at room temperature for 15 min. The mixture was treated in the same way as 2NFPH to give DNFPH as yellow needles (mp 140.5 – 141.5˚C). ESI-MS; *m*/*z* 265.0 $[M–H]^-$ (100%).

Derivatization of 24,25(OH)$_2$D$_3$ with boronic acid derivatives

The standard sample or human plasma extract was dissolved in pyridine (50 µL) containing a boronic acid derivative (5 µg) and the mixture was kept at 50˚C for 1 h. After removal of solvent, the product was dissolved in MeCN, an aliquot of which was subjected to LC/MS.

Derivatization of oxosteroids with hydrazine derivatives

To a solution of the standard oxosteroids or human plasma extract in EtOH (20 µL), a freshly prepared solution of a hydrazine derivative (5 µg) in MeCN (50 µL) containing 0.5 mg of trichloroacetic acid was added, and the mixture was kept at 60˚C for 1 h. After removal of solvents, the product was dissolved in EtOH, an aliquot of which was subjected to LC/MS.

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 3]. The ions listed in Tables 1, 2 or 3 were monitored in the mass chromatographic mode. The scan ranges were ±10 units of each monitoring ion, except for intact 24,25(OH)$_2$D$_3$ analysis, where *m*/*z* 370 – 430 was scanned.

Boronates: Twenty picograms of 24,25(OH)$_2$D$_3$ was derivatized

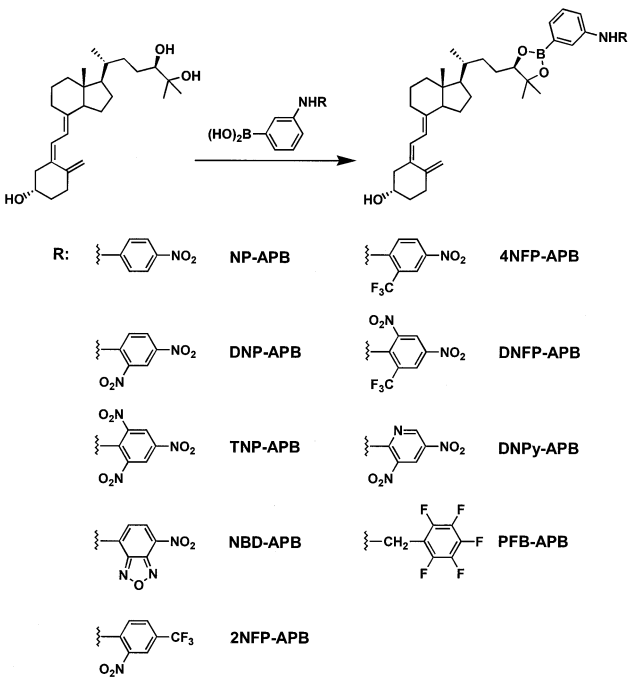

Fig. 1 Derivatization of $24,25(OH)_2D_3$ with electron-capturing boronic acid derivatives.

as described above. These derivatives were dissolved in MeCN (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 3 was determined. The LOD of the intact $24,25(OH)_2D_3$ was determined using a solution of 20 ng mL^{-1} in the same way.

Hydrazones: Two hundred picograms of oxosteroids were derivatized, dissolved in EtOH (100 μL) and then subjected to LC/MS. The amount of derivative giving an *S*/*N* of 3 was determined as described above. The LODs of the intact oxosteroids were determined using a solution of 20 ng mL^{-1}.

Pretreatment procedure for analysis of 24,25(OH)$_2$D$_3$ in human plasma

The human plasma (50 μL) obtained from a healthy male volunteer was added to MeCN (100 μL) containing [$^2H_6$]-$24,25(OH)_2D_3$ (200 pg), vortex-mixed for 30 s and centrifuged at 1500*g* (4°C, 10 min). The supernatant was diluted with H_2O (500 μL) and passed through an OASIS HLB cartridge. After successive washing with H_2O (2 mL), MeOH–H_2O, 7:3 (v/v) (2 mL) and hexane (1 mL), $24,25(OH)_2D_3$ and [$^2H_6$]-$24,25(OH)_2D_3$ were eluted with AcOEt (1 mL). After evaporation, the residue was subjected to derivatization with 2NFP-APB as described above. The resulting derivative was dissolved in MeCN (40 μL), 10 μL of which was subjected to LC/MS.

Pretreatment procedure for analysis of oxosteroids in human plasma

The human plasma (200 μL) obtained from a healthy male volunteer was added to MeCN (200 μL), vortex-mixed for 30 s and centrifuged at 1500*g* (4°C, 10 min). The supernatant was diluted with H_2O (2 mL) and then passed through an OASIS HLB cartridge. After washing with H_2O (2 mL) and MeOH–H_2O, 7:3 (v/v) (2 mL), the steroids were eluted with AcOEt (1 mL). After evaporation, the residue was dissolved in hexane–AcOEt, 3:1 (v/v) (0.2 mL × 2) and applied to a Bond Elut Si cartridge. After washing with hexane (3 mL) and hexane–AcOEt, 3:1 (v/v) (3 mL), the steroids were eluted with AcOEt–hexane, 3:1 (v/v) (3 mL). After evaporation, the residue was subjected to derivatization with 2NFPH as described above. The resulting derivative was dissolved in EtOH (40 μL), 10 μL of which was subjected to LC/MS.

Results and Discussion

Effect of derivatization with boronic acid derivatives for the detection responses in 24,25(OH)$_2$D$_3$ analysis

We first sought to find an effective electron-capturing substituent for increasing sensitivity in negative APCI-MS. $24,25(OH)_2D_3$, one of the major metabolites of vitamin D_3 expected as a new anti-osteoporosis medicine, was used as a model analyte. Conventional LC/ESI- or APCI-MS does not demonstrate sufficient sensitivity for analysis of $24,25(OH)_2D_3$ in plasma (normal range: 0.5 – 2 ng mL^{-1}), due to its low ionization efficiency. As mentioned above, we have developed a practical LC/positive APCI-MS method for the determination of the metabolite in human plasma as the derivative with the proton-affinitive Cookson-type reagent.[7] In the present study, negative APCI-MS of $24,25(OH)_2D_3$ was examined, which is expected to exceed the previous method in sensitivity.

$24,25(OH)_2D_3$ has the vicinal diol structure on its side chain, so that boronic acid was chosen as the reacting group, and nine derivatives shown in Fig. 1 were synthesized from APB. APB was very suitable for a starting material, because its primary amino group easily reacted with various labeling reagents for an amine to yield boronic acid derivatives having various substituents in a reasonable yield. As mentioned in the introductory section, it has been reported that the introduction of the PFB moiety increases the sensitivity 25 – 100 fold in estrogens in negative APCI-MS when compared with their intact compounds.[6] Based on this information, PFB-APB was synthesized as the reference reagent, but all other reagents have one or more nitro group(s) and some of them have an additional trifluoromethyl group.

The LC/MS conditions of intact $24,25(OH)_2D_3$ and its boronates were optimized as described in Table 1, where the mobile phases were adjusted so that their retention times (t_Rs) were between 6 and 7 min. Because intact $24,25(OH)_2D_3$ provided a very weak $[M–H]^-$ ion at *m*/*z* 415, leading to very low sensitivity in negative APCI-MS (LOD; more than 1 ng), it was analyzed in the positive-ion mode. Intact $24,25(OH)_2D_3$ ionized much more efficiently in the mobile phase using MeOH than in that using MeCN.

$24,25(OH)_2D_3$ was treated with the boronic acid derivatives and the resulting boronates were subjected to LC/MS without removing the excess reagents. Due to susceptibility of the boronates to solvolysis, they have generally been used in normal-phase LC.[10] However, Gamoh and co-workers have demonstrated the utility of boronates for characterization of 1,2-diol compounds in reversed-phase LC[11] and LC/MS.[12] We also found that the boronates examined in this study were remarkably stable in the mobile phase using MeCN, though they were solvolyzed in aqueous MeOH. Intact $24,25(OH)_2D_3$ gave a protonated molecular ion together with its intense dehydrated ions in positive APCI-MS, whereas all the boronates, except for that with PFB-APB, gave very intense molecular anions only in negative APCI-MS (Table 1). This demonstrated that the boronates underwent electron capture in the APCI source as observed in the study of Singh *et al.*[6] That is, if conventional APCI had occurred, then $[M–H]^-$ ions would have been

observed. In fact, when derivatives were analyzed by positive APCI, the expected mass spectra were obtained, in which $[M + H]^+$ ions were observed as major ions. In contrast, the PFB-APB derivative gave an ion at *m*/*z* 709, which may be the $[M + O_2–HF]^-$ ion, but the exact identification has not been done. Thus, for the PFB-APB derivative, we could not confirm whether dissociative electron capture had occurred.

The ions listed in Table 1 were used as monitoring ions to evaluate the effect of the derivatization for sensitivity. Although the detection response of the derivative with PFB-APB was increased by 32-fold over intact $24,25(OH)_2D_3$, all other boronates having nitro group(s) showed higher sensitivity. The effect of 2NFP-APB was particularly impressive; an injection of about 1 fmol of boronates [equivalent to about 0.4 pg of $24,25(OH)_2D_3$] was readily detected. Of course, this effect was largely caused by the high electron-affinity of the nitro group, but other factor may contribute to increase the sensitivity, judging from the fact that the magnitude of the increase of the sensitivity in similar boronates having one nitro group (the derivatives with NP-, NBD- and 4NFP-APB) were not as large as that in the derivative with 2NFP-APB. It appears that the introduction of a trifluoromethyl group does not always influence the sensitivity, because the derivatives with NP- and 4NFP-APB showed almost equal sensitivity. We supposed that the use of the unusual mobile phase significantly contributed to increase the sensitivity in the derivative with 2NFP-APB. That is, because the 2NFP-APB derivative was much strongly retained on the C18 column, the mixture of MeCN and MeOH not containing water was employed as the mobile phase to elute the derivative with an adequate t_R. In ECAPCI-MS, molecular ion, $[M]^-$, is formed with low-energy electrons through electron capture.[6] Therefore, contrary to positive APCI-MS requiring a proton-releasing solvent, such as water, the mobile phase without water can be used in ECAPCI-MS, and an analyte may be more easily desolvated and efficiently ionized in such a mobile phase than in a water-rich mobile phase. Indeed, as compared among regio-isomers, the 4NFP-APB derivative was inferior to the 2NFP-APB derivative in the sensitivity (one sixth), because the mobile phase of the former contained water.

The boronates having two nitro groups (the derivatives with DNP-, DNFP- and DNPy-APB) also gave good results with LODs of less than 2 fmol; those were more than 100 times superior to that obtained without derivatization. However, the detection response of the TNP-APB derivative was about half of that of the DNP-APB derivative, which indicates that the more nitro groups do not always increase the sensitivity.

Analysis of 24,25(OH)$_2$D$_3$ in human plasma

The application of 2NFP-APB, the most sensitive labeling reagent in negative APCI-MS analysis of $24,25(OH)_2D_3$, to the determination of its plasma levels was examined. In the previous method using the Cookson-type reagent,[7] LOD was 18 fmol and 300 μL of plasma was required for the analysis. Furthermore, because some endogenous substances lowered the derivatization rate, the method required two steps of chromatographic purifications to remove them prior to derivatization. The negative APCI-MS of $24,25(OH)_2D_3$ using 2NFP-APB provided the lower LOD (1 fmol), so that it was thought that its plasma levels could be measured with a smaller sample volume and a simpler pretreatment procedure.

Fifty microliters of plasma, which was the smallest volume used in hitherto developed methods for $24,25(OH)_2D_3$ assays,[1] was extracted with MeCN, purified with one disposable cartridge, derivatized and subjected to LC/MS analysis. A typical chromatogram is shown in Fig. 3a, in which the peak

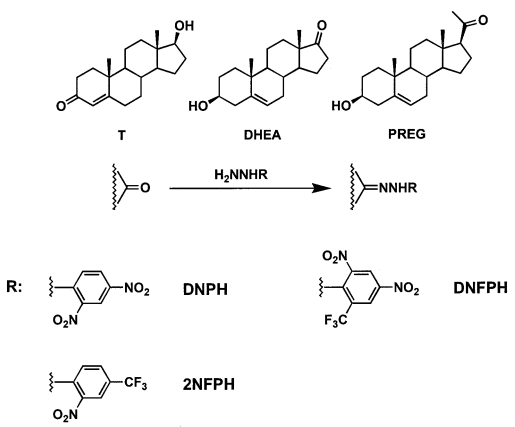

Fig. 2 Derivatization of oxosteroids with electron-capturing hydrazine derivatives.

corresponding to the $24,25(OH)_2D_3$ derivative was clearly observed at 6.9 min (about 1.8 ng mL^{-1}; calculated from the calibration curve described below). $[^2H_6]$-$24,25(OH)_2D_3$ spiked to plasma sample (*m*/*z* 712, t_R 6.8 min) was simultaneously analyzed because it will be employed as the internal standard in future quantitative assays. Strange to say, the peak area of the $[^2H_6]$-$24,25(OH)_2D_3$ derivative was about half as that of $24,25(OH)_2D_3$, which may be caused by the difference of ionization efficiencies of the both compounds. That is, the slope of the regression line (10, 20, 50, 100 and 200 pg of $24,25(OH)_2D_3$ and 200 pg of $[^2H_6]$-$24,25(OH)_2D_3$ per tube) was 2.15, where the *x* axis was the amount ratio $\{24,25(OH)_2D_3/[^2H_6]\text{-}24,25(OH)_2D_3\}$ and the *y* axis was the peak area ratio $\{24,25(OH)_2D_3/[^2H_6]\text{-}24,25(OH)_2D_3\}$, though good linearity was obtained in the range (correlation coefficient $r^2 = 0.999$). MS/MS analysis with 25% of relative collision energy was also performed to confirm the purity of the peak at 6.9 min, in which the molecular ion (*m*/*z* 706.3) was used as the precursor ion. The product ion mass spectrum of plasma sample (Fig. 3b) was completely identical with that of standard sample, though the assignment of the product ions could not be fully done. These data indicate that the present method is highly sensitive and has enough selectivity.

Effect of derivatization with hydrazine derivatives for the detection responses in oxosteroids analyses

On the basis of the data obtained from the study on boronic acid derivatives for $24,25(OH)_2D_3$ analysis, three hydrazine derivatives having the DNP, 2NFP or DNFP moiety were purchased or synthesized for derivatization reagents of oxosteroids (Fig. 2). PREG was used as a model compound and reacted with these hydrazines for conversion to the corresponding hydrazones. The LOD of the formed hydrazones were examined in a similar manner as done for the $24,25(OH)_2D_3$-boronates (Table 2). All the hydrazones provided only their molecular ions, $[M]^-$, in negative APCI-MS. The highest sensitivity was obtained for PREG-2NFPH, which was in accord with expectations based on the data obtained in the $24,25(OH)_2D_3$-boronate study. However, the magnitude of the increase of the sensitivity by introduction of the 2NFP moiety in the PREG (17-fold) was much smaller than that observed in $24,25(OH)_2D_3$ (225-fold). On the whole, the boronic acid derivatives for $24,25(OH)_2D_3$ were much more effective than the hydrazine derivatives for PREG, if the derivatization reagents had the same electron-capturing

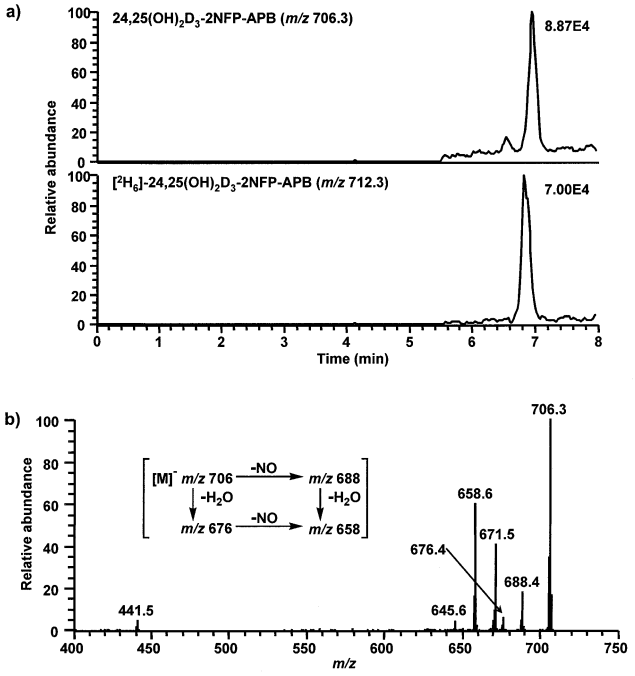

Fig. 3 LC/MS data of 24,25$(OH)_2D_3$ in human plasma as derivative with 2NFP-APB. a) Mass chromatograms of derivatized 24,25$(OH)_2D_3$ (*m*/*z* 706.3) and [$^2H_6$]-24,25$(OH)_2D_3$ (*m*/*z* 712.3). b) Product ion mass spectrum of derivatized 24,25$(OH)_2D_3$ (t_R, 6.9 min). LC/MS conditions were the same as those of the 2NFP-APB derivative in Table 1.

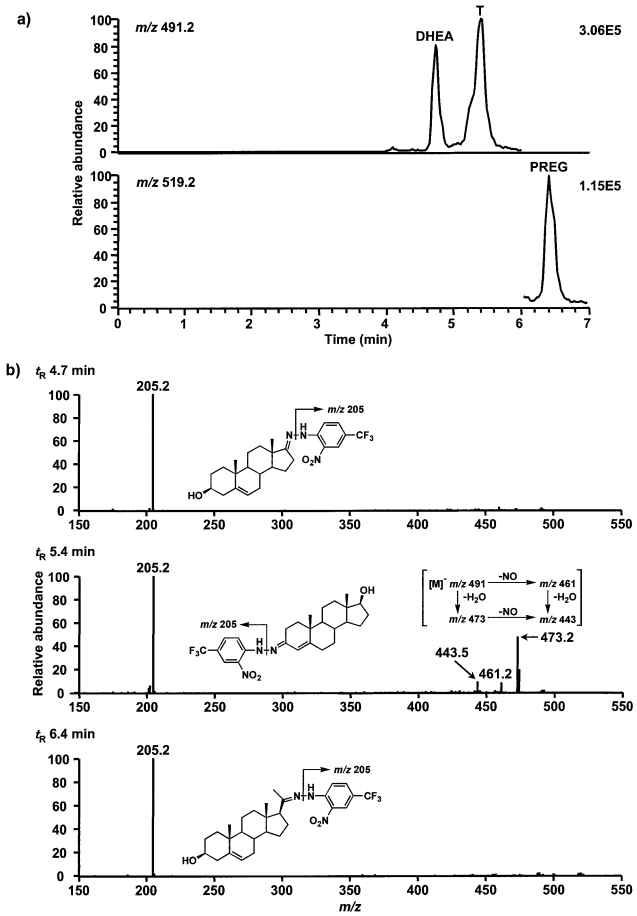

Fig. 4 LC/MS data of oxosteroids in human plasma as derivatives with 2NFPH. a) Mass chromatograms of derivatized oxosteroids. b) Product ion mass spectra of derivatized oxosteroids. LC/MS conditions were the same as those of the 2NFPH derivative of PREG in Table 2.

substituents (Tables 1 and 2).

Next, the relationship between the effect of derivatization with 2NFPH in increasing sensitivity and location of the carbonyl group in oxosteroids was studied. The oxosteroids were grouped into three subclasses: 3-oxo-Δ^4-steroid, 17-oxosteroid and 20-oxosteroid, and T, DHEA and PREG were used as their model compounds, respectively. The LODs of derivatized steroids were compared with their intact steroids in a similar manner as above. The results are summarized in Table 3. DHEA and PREG (3β-hydroxysteroids) showed extensive water loss, whereas T (3-oxo-Δ^4-steroid) produced a stable protonated molecular ion as the base ion; these results were compatible with the report by Kobayashi *et al.*[13] The LODs of intact steroids varied widely and T provided relatively low LOD, which was below one fifth of that of DHEA. The derivatization with 2NFPH was seemingly most effective in DHEA (37-fold), but it is due to the large LOD of intact DHEA, and the LOD of the derivatized DHEA was not significantly different from that of the derivatized T or PREG (10 – 16 fmol, equivalent to 3 – 5 pg of intact steroids). Incidentally, derivatization rates of each of the oxosteroids were inferred to be almost quantitative, because intact oxosteroids were not detected in LC/MS after the derivatization reaction.

Analysis of neutral oxosteroids in human plasma

In the 1980s, Baulieu and co-workers demonstrated that some oxosteroids, such as PREG, DHEA and their sulfates, are present in higher concentrations in the brain than in blood and are synthesized *de novo* in the central nervous system.[14] Such steroids are now universally referred to as neurosteroids. They may act as modulators of several membrane receptors either as stimulators or inhibitors[15] and be involved in learning and memory performance.[16] To date, various oxosteroids, such as T, are dealt with as neurosteroids or neuroactive steroids, though they are either synthesized in the brain or in the peripheral organs, but with an action on neuronal tissue.[17,18] For these reasons, there is a considerable interest in determining the levels of different oxosteroids in the body fluids and tissue. GC/MS has been conventionally used for this purpose,[17,18] but LC/ESI-MS combined with derivatization has recently been examined for analysis of these steroids.[3,4]

The utility of 2NFPH as the derivatization reagent for the analysis of oxosteroids with negative APCI-MS, that is, the profile analysis of DHEA, PREG and T in the plasma (some nanograms per milliliter in a normal subject), was studied. A plasma sample was deproteinized with MeCN, purified with two disposable cartridges, derivatized and subjected to LC/MS analysis (Fig. 4a). Comparison with t_Rs of reference compounds indicated that the peaks at 4.7 and 5.4 min in the reconstructed ion chromatograms of *m*/*z* 491.2 corresponded to DHEA and T derivatives, respectively, and that at 6.4 min in the chromatogram of *m*/*z* 519.2 corresponded to the PREG derivative. Due to the formation of *syn* and *anti* isomers, the T derivative gave a broad peak. All the peaks corresponding to the T, DHEA and PREG derivatives produced an intense product ion at *m*/*z* 205 in the MS/MS analysis (relative collision energy 20%), which was assigned as the 2NFPH-NH moiety (cleavage of the N–N bond of the hydrazone) (Fig. 4b). Product ion mass spectra of these peaks were identical with those of

authentic samples.

Conclusions

The derivatization reagents having electron-capturing moieties, such as nitro group(s), were designed and synthesized for negative APCI-MS analysis of neutral steroids. The derivative with 2NFP-APB provided over 200-fold higher sensitivity compared to intact 24,25$(OH)_2D_3$, where the LOD was below 1 fmol. The LODs of some picograms were also obtained for the analyses of oxosteroids by derivatization with 2NFPH. Thus, the 2NFP moiety has an outstanding ability of electron-capture and the introduction of it to analytes is very effective in enhancing the sensitivity in negative APCI-MS. These results also demonstrate that many kinds of sensitive derivatization reagents for various biomolecules and drugs can be synthesized by combination of the 2NFP moiety and other reacting groups. In addition to the syntheses of new derivatization reagents, clinical and biological sample analyses using 2NFP-APB and 2NFPH, such as the determination of neurosteroids in the brain, are now in progress in our laboratories.

Acknowledgements

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