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Simple and practical derivatization procedure for enhanced detection of carboxylic acids in liquid chromatography–electrospray ionization-tandem mass spectrometry

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ABSTRACT

A simple and practical derivatization procedure for increasing the detection responses of carboxylic acids in liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) has been developed. 2-Hydrazinopyridine (HP) and 2-picolylamine (PA) rapidly reacted with biologically and clinically important carboxylic acids [chenodeoxycholic acid, glycochenodeoxycholic acid, prostaglandin E_2, 2-(β-carboxyethyl)-6-hydroxy-2,7,8-trimethylchroman (γ-CEHC), α-lipoic acid, homovanillic acid (HVA) and 5-hydroxyindole-3-acetic acid] in the presence of 2,2′-dipyridyl disulfide and triphenylphosphine. The resulting HP- and PA-derivatives were highly responsive in ESI-MS operating in the positive-ion mode and gave characteristic product ions during MS/MS, which enabled the sensitive detection using selected reaction monitoring. Among the two reagents, PA was of more practical use; the detection responses of the PA-derivatives were increased by 9–158-fold over the intact carboxylic acids and the limits of detection were in the low femtomole range (1.5–5.6 fmol on column). The PA-derivatization was successfully applied to a biological sample analysis; the derivatization followed by LC–ESI-MS/MS enabled the detection of trace amounts of bile acids, γ-CEHC and HVA in human saliva with a simple pretreatment, small sample volume and short analysis time.

1. Introduction

Compounds having a carboxyl group, which are simply described as carboxylic acids in this paper, are widely distributed in nature and play important roles at trace levels in the regulation of a variety of physiological and biological functions. Therefore, the characterization and determination of carboxylic acids in biological fluids and tissues are often of clinical value, especially in the elucidation of the nature, diagnosis and treatment of a number of metabolic and nutrition disorders. Because of the metabolic versatility and their occurrence at low concentrations in body fluids and tissues, the development of a reliable analytical method of carboxylic acids is a challenging subject for analytical biochemists.

Numerous methods have been described for the characterization and determination of carboxylic acids, such as high-performance liquid chromatography (HPLC) [1,2] and gas chromatography–mass spectrometry (MS) [2]. Recently, liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) has been frequently used for the analysis of physiologically important carboxylic acids due to its high specificity and versatility [3]. Carboxylic acids are ionized by ESI-MS operating in the negative-ion mode using a basic mobile phase in which carboxyl groups are ionized, but the negative ESI-MS/MS sometimes does not demonstrate the required sensitivity for the trace analysis of carboxylic acids. One of the reasons for this is that only a nonspecific and/or low-intensity product ion is sometimes formed by collision induced dissociation (CID) of a negatively charged ion, and as a result, selected reaction monitoring (SRM), the highly specific technique available using a triple quadrupole mass spectrometer, cannot be employed. In fact, some fatty acids were analyzed using the selected ion monitoring mode due to the failure to obtain their characteristic product ions by MS/MS [4]. Furthermore, for carboxylic acid analysis, the best chromatographic resolution with reversed-phase columns is achieved at an acidic pH where the ionization of the carboxyl groups is suppressed.

To enhance the detection responses of carboxylic acids in ESI-MS/MS, several chemical derivatization procedures suitable for the positive-ion detection have been developed. For example, tris(2,4,6-trimethoxyphenyl)phosphonium propylamine (TMPP) bromide was used as the derivatization reagent for the LC–ESI-

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MS/MS analysis of carboxylic acids in pharmaceutical products [5]. A TMPP-derivatized carboxylic acid has a significantly high molecular mass (increase by 572 Da over the molecular mass of an intact carboxylic acid). It is common knowledge that a larger reagent has the advantage that its derivatized product has a high mass and thus will enable a signal to be observed in the less noisy region of the mass spectrum. However, when using a triple quadrupole instrument, which is the most widely used one for quantitative analysis, the shift of the mass of the analyte to a higher mass range is not always preferable for the sensitivity; in fact, our instrument (Applied Biosystems API 2000) often does not demonstrate a sufficient sensitivity for compounds with molecular masses over 800.

Santa and co-workers developed 4-[2-(*N*,*N*-diethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE, molecular mass, 328 Da) as a novel derivatization reagent for the LC–ESI-MS/MS of carboxylic acids [6]. The derivatives with DAABD-AE had a high proton-affinity and provided a characteristic product ion during MS/MS. This reagent was successfully applied to the LC–ESI-MS/MS analysis of dicarboxylic acids [7] and very long chain fatty acids [8] for the diagnosis of glutaric acidemia and peroxisomal disorders, respectively. As a disadvantage of this reagent, it is not currently commercially available, which sometimes becomes a problem for routine assays.

The conversion of a carboxylic acid into the trimethylaminoethyl (TMAE) ester (increase by 86 Da over the molecular mass of an intact carboxylic acid) is also used for its sensitive analysis [3,9]. This procedure uses commercially available reagents, but requires three reaction steps (i.e., conversion of a carboxylic acid into its acyl chloride, dimethylaminoethyl-esterification and quaternarization) and is complicated.

Based on this background information, in this study, we examined simple derivatization procedures using commercially available and small reagents for increasing the sensitivity and specificity of carboxylic acids in the positive ESI-MS/MS. The carboxylic acids shown in Fig. 1 were biologically and clinically important and were examined as the model compounds in this study. The application of the developed procedure for the detection of trace amounts of some carboxylic acids in saliva is also described.

2. Materials and methods

2.1. Chemicals and reagents

Chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid (GCDCA), α-lipoic acid (α-LA), cholic acid (CA) and 4-hydroxy-3-methoxyphenylpropionic acid (HMPP) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Prostaglandin E_2 (PGE_2) and 5-hydroxyindole-3-acetic acid (5-HIAA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Homovanillic acid (HVA) was obtained from Kanto Chemical (Tokyo). 2-(β-Carboxyethyl)-6-hydroxy-2,7,8-trimethylchroman (γ-CEHC) was kindly supplied by Eisai (Tokyo). [2,2,4,4-$^2H_4$]-CDCA (D_4-CDCA) was obtained from CDN Isotopes (Quebec, Canada). (*S*)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich Japan (Tokyo). Stock solutions of the carboxylic acids were prepared as 100 μg/ml solutions in methanol (CDCA, GCDCA, α-LA, CA and D_4-CDCA) or ethanol (PGE_2, γ-CEHC, HVA, 5-HIAA, Trolox and HMPP). Subsequent dilutions were carried out with methanol or ethanol to prepare 1.0, 10, 100 and 1000 ng/ml solutions. The reagents used for the derivatization, 2-hydrazinopyridine (HP), 2-picolylamine (PA), 2,2′-dipyridyl disulfide (DPDS) and triphenylphosphine (TPP), were purchased from Tokyo Chemical

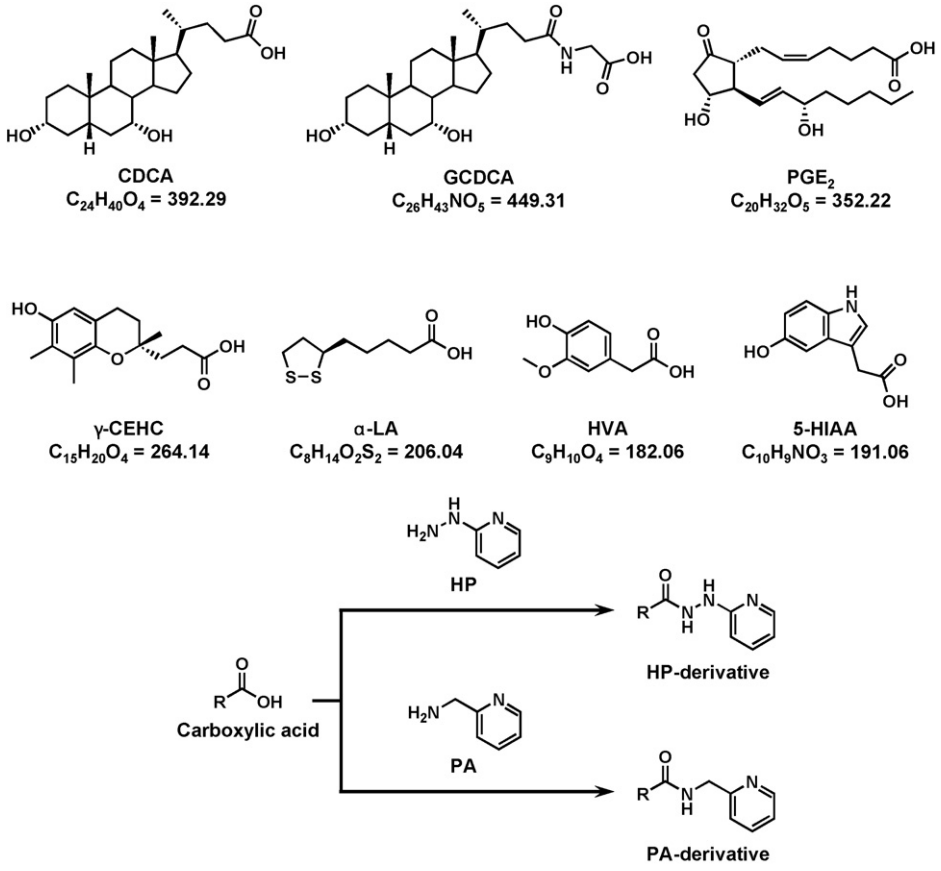

Fig. 1. Chemical structures of carboxylic acids and their HP- and PA-derivatives.

Table 1
Optimized MS conditions for intact and derivatized carboxylic acids.

	Polarity	DP (V)	FP (V)	IS (V)	CUR (psi)	GAS 1 (psi)	CE (eV)
CDCA (intact)	Negative	−70	−310	−4000	55	40	−50
CDCA-HP	Positive	50	200	4000	45	80	50
CDCA-PA	Positive	55	350	4000	45	80	65
GCDCA (intact)	Negative	−70	−310	−4000	55	40	−50
GCDCA (intact)	Positive	50	300	4500	45	80	45
GCDCA-HP	Positive	50	200	4000	45	80	50
GCDCA-PA	Positive	55	350	4000	45	80	65
PGE_2 (intact)	Negative	−15	−250	−4500	45	80	−20
PGE_2-HP	Positive	15	380	4000	45	80	35
PGE_2-PA	Positive	40	380	4500	45	80	40
γ-CEHC (intact)	Negative	−30	−360	−3000	55	40	−20
γ-CEHC-HP	Positive	20	350	5000	45	80	40
γ-CEHC-PA	Positive	20	380	4000	45	80	40
α-LA (intact)	Negative	−10	−300	−4000	45	80	−15
α-LA-HP	Positive	30	380	4500	45	80	30
α-LA-PA	Positive	25	380	4000	45	80	35
HVA (intact)	Negative	−20	−350	−4000	45	80	−20
HVA-HP	Positive	30	200	5000	45	80	30
HVA-PA	Positive	30	380	4000	45	80	30
5-HIAA (intact)	Negative	−15	−350	−4000	50	80	−15
5-HIAA (intact)	Positive	90	200	4000	55	80	35
5-HIAA-HP	Positive	15	380	3000	55	80	25
5-HIAA-PA	Positive	20	380	4000	50	80	30

Industry. A Strata-X cartridge (60 mg adsorbent; Phenomenex, Torrance, CA, USA) was used for the solid-phase extraction of the saliva sample. All other reagents and solvents were of analytical grade.

2.2. LC–MS/MS

LC–MS/MS was performed using an Applied Biosystems API 2000 triple quadrupole mass spectrometer (Foster City, CA) connected to a Shimadzu LC-20AD chromatograph (Kyoto, Japan). 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. The HP- and PA-derivatives of the carboxylic acids were analyzed by ESI-MS in the positive-ion mode, and the intact carboxylic acids were analyzed in the negative-ion mode unless otherwise noted. The intact GCDCA and 5-HIAA were also analyzed in the positive-ion mode. The MS conditions were as follows: entrance potential (EP), ±10 V; ion source gas 2 (GAS 2, nitrogen), 80 psi; turbo gas temperature, 500 °C and interface heater, on. Nitrogen was used as the collision gas in the SRM mode with a collision gas of 6 (arbitrary unit) and a collision cell exit potential (CXP) of ±10 V. The declustering potential (DP), focusing potential (FP), ion spray voltage (IS), curtain gas (CUR, nitrogen), ion source gas 1 (GAS 1, nitrogen) and collision energy (CE) for each analyte are described in Table 1. The mobile phases (isocratic elution) and transitions (precursor and product ions) for each analyte are described in Table 2.

Table 2
Mobile phase, transition for SRM and LOD for intact and derivatized carboxylic acids.

	Mobile phase[a]	t_R (min)	Transition (precursor ion, m/z/product ion, m/z)	LOD (fmol)	Increasing sensitivity[b]
CDCA (negative)	A (17:3)	3.6	391.2 $[M-H]^-$/no characteristic product ion	–[c]	–[c]
CDCA-HP	B (7:3) cont. 0.1% (v/v) HCOOH	4.0	484.5 $[M+H]^+$/110.1 $[HP+H]^+$	5.1	–[c]
CDCA-PA	B (4:1) cont. 0.1% (v/v) HCOOH	4.1	483.4 $[M+H]^+$/109.3 $[PA+H]^+$	2.5	–[c]
GCDCA (negative)	A (3:1)	4.0	448.3 $[M-H]^-$/74.1 $[glycine-H]^-$	49 (22)[d]	1
GCDCA (positive)	B (4:1) cont. 0.1% (v/v) HCOOH	4.2	414.5 $[M+H-2H_2O]^+$/76.3 $[glycine+H]^+$	110	0.4
GCDCA-HP	B (7:3) cont. 0.1% (v/v) HCOOH	3.7	541.5 $[M+H]^+$/110.1 $[HP+H]^+$	6.7	7
GCDCA-PA	B (7:2) cont. 0.1% (v/v) HCOOH	4.1	540.5 $[M+H]^+$/109.3 $[PA+H]^+$	5.6	9
PGE_2 (negative)	A (7:4)	3.8	351.1 $[M-H]^-$/271.0 $[M-H-CO_2-2H_2O]^-$	71 (25)[d]	1
PGE_2-HP	A (2:1)	4.2	444.4 $[M+H]^+$/110.2 $[HP+H]^+$	5.7	12
PGE_2-PA	A (2:1)	4.3	425.5 $[M+H-H_2O]^+$/109.3 $[PA+H]^+$	2.8	25
γ-CEHC (negative)	A (11:9)	3.7	263.0 $[M-H]^-$/218.9 $[M-H-CO_2]^-$	300 (80)[d]	1
γ-CEHC-HP	A (3:2)	4.2	356.4 $[M+H]^+$/110.2 $[HP+H]^+$	4.5	67
γ-CEHC-PA	A (7:4)	3.7	355.4 $[M+H]^+$/109.3 $[PA+H]^+$	1.9	158
α-LA (negative)	A (11:9)	3.6	204.9 $[M-H]^-$/170.9 $[M-H-H_2S]^-$	97 (20)[d]	1
α-LA-HP	A (7:4)	3.8	298.3 $[M+H]^+$/110.2 $[HP+H]^+$	2.9	33
α-LA-PA	A (7:4)	4.0	297.3 $[M+H]^+$/109.3 $[PA+H]^+$	1.5	65
HVA (negative)	B (7:13) cont. 0.005% (v/v) CH_3COOH	4.4	181.1 $[M-H]^-$/121.9 $[M-H-CO_2-CH_3]^-$	190 (35)[d]	1
HVA-HP	A (7:13)	3.6	274.1 $[M+H]^+$/110.1 $[HP+H]^+$	11	17
HVA-PA	A (7:13)	4.0	273.1 $[M+H]^+$/109.2 $[PA+H]^+$	2.2	86
5-HIAA (negative)	B (3:7) cont. 0.005% (v/v) CH_3COOH	3.8	190.1 $[M-H]^-$/146.1 $[M-H-CO_2]^-$	79 (15)[d]	1
5-HIAA (positive)	B (1:3) cont. 0.1% (v/v) HCOOH	3.8	146.1 $[M+H-HCOOH]^+$/90.9 (not assigned)	79	1
5-HIAA-HP	A (3:7)	3.6	283.0 $[M+H]^+$/110.2 $[HP+H]^+$	10	8
5-HIAA-PA	A (3:7)	3.9	282.0 $[M+H]^+$/109.3 $[PA+H]^+$	2.6	30

[a] A: mixture of methanol and 10 mM ammonium formate (v/v); B: mixture of methanol and water (v/v). For example, B (7:3) cont. 0.1% (v/v) HCOOH means methanol:water (7:3, v/v) containing 0.1% (v/v) formic acid.
[b] The detection responses of intact carboxylic acids (negative-ion mode) are taken as 1.
[c] Because no characteristic product ion was formed, the LOD and the increase of the sensitivity were not determined.
[d] The values in parentheses are the amounts converted from fmol unit to pg unit.

Analyst software (version 1.3.1, Applied Biosystems) was used for the system control and data processing.

2.3. Derivatization of carboxylic acids with HP (optimized condition)

To a carboxylic acid, a freshly prepared solution of TPP (10 mM) in acetonitrile (10 μl), DPDS (10 mM) in acetonitrile (10 μl) and HP (10 μg) in acetonitrile (10 μl) were successively added, and the mixture was stored at 60 °C for 10 min. After removal of the solvent, the product was dissolved in the mobile phase, an aliquot of which was subjected to LC–MS/MS.

2.4. Derivatization of carboxylic acids with PA (optimized condition)

The standard carboxylic acid or the pretreated saliva sample was derivatized using the same procedure of HP-derivatization with the substitution of PA [10 μg in acetonitrile (10 μl)] for HP. For the saliva analysis, the product was dissolved in the mobile phase (45 μl), 15 μl of which was subjected to LC–MS/MS.

2.5. Optimization of reaction time and temperature in derivatization

γ-CEHC or HVA (100 pg) was derivatized with HP at room temperature or 60 °C for 5, 10 or 30 min. After the prescribed reaction time, methanol–acetic acid (99:1, v/v, 10 μl) was added to the reaction mixture to terminate the reaction. The HP-derivative of Trolox (100 pg) or HMPP (100 pg), which had been previously prepared, was also added to the mixture. After evaporation of the solvents, the residue was dissolved in the mobile phase for γ-CEHC-HP or HVA-HP (45 μl, Table 2), 15 μl of which was subjected to LC–MS/MS. The peak area ratios of γ-CEHC-HP/Trolox-HP or HVA-HP/HMPP-HP under the respective reaction conditions were compared.

The optimal reaction time and temperature for the PA-derivatization were also examined in the same way as the HP-derivatization.

The SRM transitions for Trolox-HP, Trolox-PA, HMPP-HP and HMPP-PA were m/z 342.3 → 205.1, m/z 341.3 → 205.1, m/z 288.1 → 110.1 and m/z 287.1 → 109.2, respectively.

2.6. 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 transitions listed in Table 2 were monitored in the SRM mode. The carboxylic acids (100 pg) were derivatized with HP or PA as described above. These derivatives were dissolved in the mobile phases (200 μl) listed in Table 2 and then subjected to LC–MS/MS. By stepwise decreasing the injection volume of the resulting solution, the amount of the derivative giving an S/N of 5 was determined. The LODs of the intact carboxylic acids were determined using a solution of 10 ng/ml in the same way.

2.7. Collection and pretreatment of saliva sample

Saliva (ca. 1 ml) was directly collected into a collecting tube (without a collection device) from healthy subjects and stored at −15 °C until use. The subjects ingested no food and beverage within 30 min prior to the sample collection. The subjects also did not brush their teeth within 1 h prior to the sample collection to avoid any blood contamination. The saliva sample was centrifuged at 1000 × g for 10 min prior to use. Written informed consent was obtained from all the subjects. The experimental procedures were conducted in accordance with the ethical standards of the Helsinki Declaration and approved by the Ethics Committee of the University of Shizuoka.

2.7.1. Pretreatment for analysis of primary non-amidated bile acids in saliva

The saliva sample (200 μl) was added to heated ethanol (500 μl, 60 °C) containing D_4-CDCA (100 pg), vortex-mixed for 30 s and centrifuged at 1000 × g for 10 min. The supernatant was diluted with 0.02 M acetic acid aqueous solution (1.5 ml), and the sample was passed through a Strata-X cartridge, which had been successively pre-washed with methanol (2 ml) and 0.02 M acetic acid (2 ml). After washing with water (2 ml), the bile acids were eluted with methanol (2 ml). After evaporation, the residue was subjected to the PA-derivatization.

2.7.2. Pretreatment for analysis of γ-CEHC in saliva

The saliva sample (200 μl) was added to acetonitrile (200 μl), vortex-mixed for 30 s and centrifuged at 1000 × g for 10 min. After concentration of the supernatant to about half volume under a N_2 gas stream, the sample was extracted with ethyl acetate (200 μl, two times). The combined ethyl acetate layer was washed with water (200 μl), and the solvent was then evaporated under a N_2 gas stream. The residue was subjected to the PA-derivatization.

2.7.3. Pretreatment for analysis of HVA in saliva

The saliva (100 μl) was diluted with a 0.02 M acetic acid aqueous solution (500 μl) containing HMPP (100 pg), and the sample was passed through a Strata-X cartridge, which had been successively pre-washed with ethyl acetate (2 ml), methanol (2 ml) and 0.02 M acetic acid (2 ml). After washing with water (2 ml) and hexane (2 ml), the HVA and HMPP were eluted with ethyl acetate (1 ml). After evaporation, the residue was subjected to the PA-derivatization.

3. Results and discussion

3.1. Derivatization of carboxylic acids with HP and PA

Some reagents have been investigated for the ESI-MS/MS of carboxylic acids and found to be valuable under certain conditions [3,5–9], but they also have some limitations as mentioned in the Introduction. To overcome these limitations, the derivatization reagent should have the following properties: (1) the reagent is commercially available, (2) the reagent has a highly proton-affinitive moiety, that is highly responsive in the positive ESI-MS, (3) the derivatized product provides a characteristic product ion (i.e., a product ion that is specific to the analyte and the structure of which can be specified) during MS/MS, (4) the molecular mass of the reagent is low; a derivatized product with a high mass is not sometimes recommended for the sensitivity, when using a triple quadrupole mass spectrometer, and (5) the derivatization quantitatively proceeds by a one-step reaction under mild conditions. To meet such requirements, HP (molecular mass, 109 Da) and PA (molecular mass, 108 Da) were chosen as the reagents. Both reagents have a highly ESI-active moiety, i.e., the pyridyl group [10–13], and reacts with carboxylic acids in the presence of a condensation agent to form the hydrazide and amide derivatives, respectively. The hydrazide and amide are well-known fragmentable moieties in CID and therefore, are expected to generate a characteristic product ion suitable for the SRM detection.

The combination of DPDS and TPP has been conventionally used for the condensation of amines and carboxylic acids [14,15]. Based on this information, these agents were used to prepare the HP- and PA-derivatives of the carboxylic acids in this study. After the treatment with these agents, carboxylic acids were reacted with

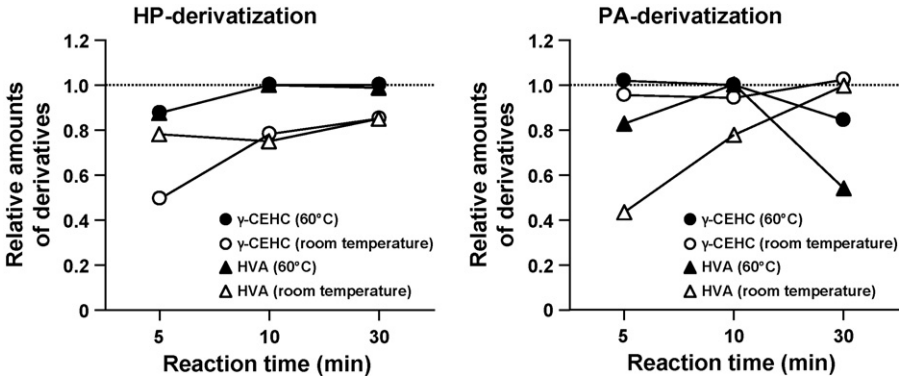

Fig. 2. Effect of reaction temperature and time on HP- and PA-derivatization. γ-CEHC and HVA were treated according to the procedure described in Section 2.5. The amounts of the derivatives formed by heating at 60 °C for 10 min were taken as 1.

a constant and excessive amount (10 μg) of the derivatization reagents (HP or PA). Acetonitrile was used as the reaction solvent according to a previous report [14]. The reaction time and temperature of the derivatization were optimized using γ-CEHC and HVA as the model compounds. The HP- and PA-derivatization reactions occurred even at room temperature (ca. 20 °C), but the reactions proceeded more rapidly at 60 °C (Fig. 2); the most abundant derivatives were formed for both γ-CEHC and HVA, when the HP- and PA-derivatization reactions were carried out at 60 °C for 10 min. During the PA-derivatization, the amounts of the derivatives, especially HVA-PA, decreased by a long heating (30 min), but all the resulting HP- and PA-derivatives were stable at room temperature for at least 1 day and at 4 °C for at least 5 days when they were dissolved in the respective mobile phases. Based on these results, heating for 10 min at 60 °C was employed for both the HP- and PA-derivatization procedures. The effect of concentrations (2, 10 and 50 mM) of the condensation agents (DPDS and TPP) on the amounts of the resulting derivatives was also examined. As a result, the derivatized products reached a plateau at concentrations of the agents over 10 mM; 10 mM was selected as optimum.

When γ-CEHC and HVA (10 ng) were derivatized with HP under the optimized condition, only negligible amounts of the underivatized γ-CEHC (190 pg, mean of duplicate assays) and HVA (410 pg) were detected; these amounts were 1.9 and 4.1%, respectively, of the initial amount (10 ng). The same experiment was performed for the PA-derivatization; the amounts of the underivatized γ-CEHC and HVA were 100 pg (1.0% of initial amount) and 140 pg (1.4% of initial amount), respectively. In this study, the absolute derivatization rates for respective carboxylic acids were not determined. However, the rates are inferred to be satisfactory (over 90%), because the amounts of γ-CEHC and HVA that remained underivatized were negligible under the optimized condition as presented above, and the carboxyl groups in other carboxylic acids are as reactive as those of γ-CEHC and HVA.

3.2. ESI-MS and -MS/MS of intact and derivatized carboxylic acids

For the ESI-MS operating in the negative-ion mode, all the intact carboxylic acids predominantly provided deprotonated molecules, $[M-H]^-$ (Table 2). The CID of $[M-H]^-$ of CDCA with a 50 eV energy produced many product ions, but none of them had a satisfactory intensity. On the contrary, when the lower collision energy was used, CDCA did not produce any product ions; the SRM mode was not applicable to the CDCA detection. The fragmentation during the CID for other carboxylic acids was variable. For PGE_2, γ-CEHC and 5-HIAA, the most abundant product ions were nonspecific ions; the loss of CO_2 (−44) from $[M-H]^-$ (γ-CEHC and 5-HIAA) and the loss of $2H_2O$ (-18×2) together with CO_2 from $[M-H]^-$ (PGE_2). The product ion of HVA corresponded to the loss of CO_2 and CH_3 (−15) from $[M-H]^-$. α-LA provided the product ion due to the loss of H_2S (−34). The product ion for GCDCA was assigned as $[glycine-H]^-$. Based on these results, the transitions shown in Table 2 were used for the determination of the LODs (S/N = 5) for the respective carboxylic acids, in which the mobile phases were adjusted so that their retention times (t_Rs) were around 4 min; the LODs of the intact carboxylic acids using the negative ESI-MS/MS (SRM) ranged from 49 to 300 fmol on the column.

Because GCDCA and 5-HIAA have a proton-affinitive nitrogen atom in the molecules, we determined the LODs for these compounds in the positive ESI-MS/MS, in which formic acid was added to the mobile phase to protonate the acids. When using the transitions shown in Table 2, their LODs in the positive-ion mode were about twice (GCDCA) or equal (5-HIAA) to those in the negative-ion mode; thus we could not find an advantage of detecting these acids as the intact forms in the positive-ion mode.

All the HP-derivatives gave intense protonated molecules, $[M+H]^+$, as the base peaks in the positive ESI-MS (Table 2). When these $[M+H]^+$ were collisionally activated, the characteristic product ions were observed at m/z 110 with satisfactory intensities for all the derivatives (Fig. 3). This product ion was formed by the cleavage of the amide bond and assigned as $[HP\ (C_5H_4NNHNH_2)+H]^+$. As mentioned above, the SRM could not be applied to the analysis of the intact CDCA (negative-ion mode), whereas the HP-derivatives of CDCA was found to be suitable for the SRM detection. The transitions listed in Table 2 were used to determine the LODs and evaluate the effect of the HP-derivatization for the detection responses. The detection responses of the derivatives were increased by 7–67-fold over the intact carboxylic acids and the LODs were in the low femtomole range. This effect was largely caused by the high proton-affinity of the pyridyl group and the characteristic fragmentation of the derivatives during MS/MS, which is quite useful in the SRM detection. The derivatization was seemingly most effective in γ-CEHC (67-fold), but this was due to the large LOD of the intact γ-CEHC, and the LOD of the derivatized γ-CEHC (4.5 fmol) was not significantly different from those of the other derivatized carboxylic acids (2.9–11 fmol).

The PA-derivatives also provided intense protonated molecules as base peaks in the positive ESI-MS, except for the PGE_2 derivative, whose base peak was $[M+H-H_2O]^+$. In MS/MS using these ions as the precursor ions, the most abundant product ions were detected at m/z 109, which corresponded to $[PA\ (C_5H_4NCH_2NH_2)+H]^+$ formed by the cleavage of the amide bonds, for all the PA-derivatized carboxylic acids (Fig. 4). The LODs of the PA-derivatives were examined in a similar manner as done for the HP-derivatives. As shown in Table 2, the PA-derivatives gave better results with LODs of 1.5–5.6 fmol on the column; the magnitude of the increase

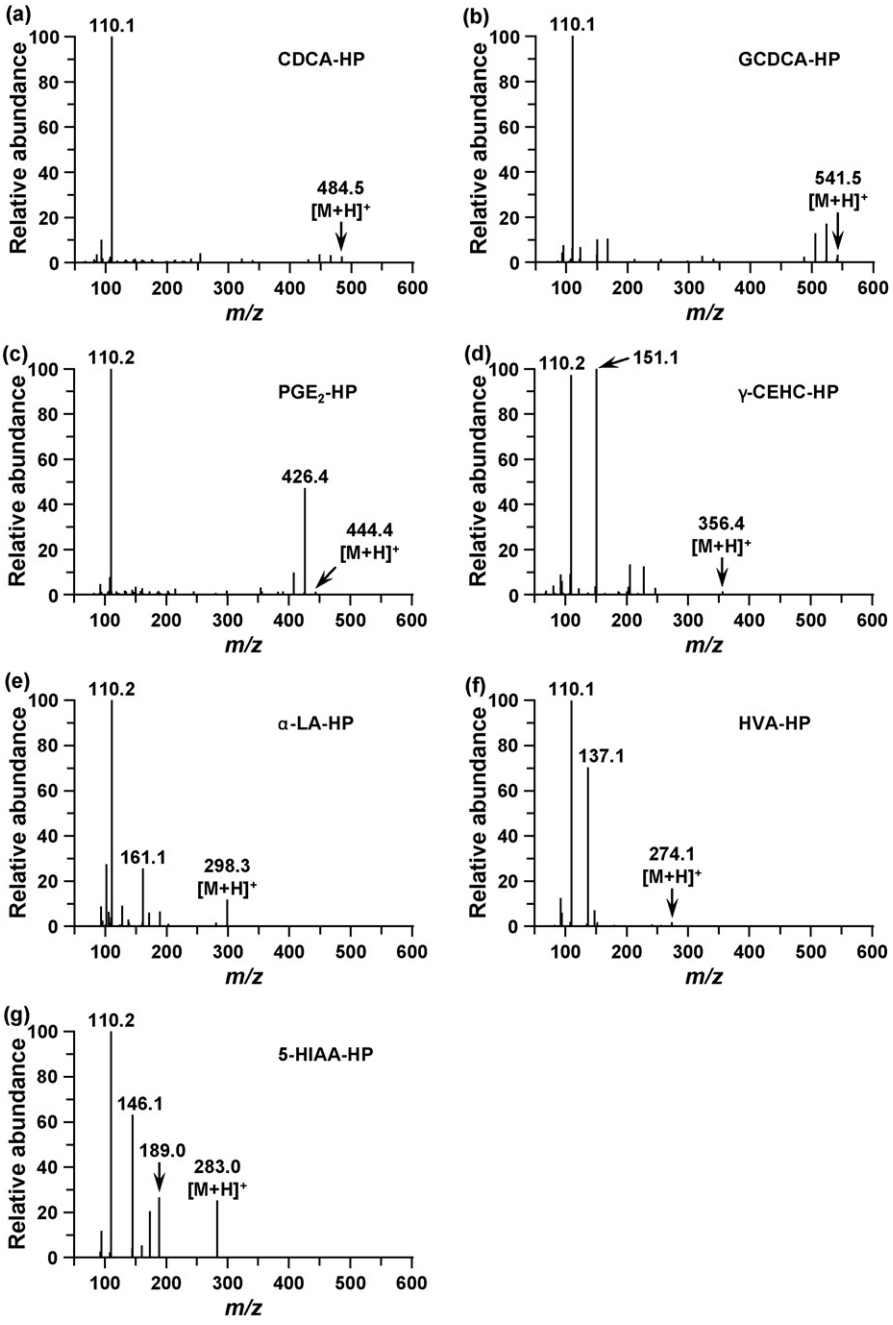

Fig. 3. ESI-MS/MS spectra of HP-derivatives of (a) CDCA, (b) GCDCA, (c) PGE_2, (d) γ-CEHC, (e) α-LA, (f) HVA and (g) 5-HIAA. The MS/MS spectra were recorded by the collisional activation of $[M+H]^+$ of the respective derivatives. The other conditions are described in Section 2.2 and Table 1.

in the detection responses by the PA-derivatization was about twice that by the HP-derivatization for CDCA, PGE_2, γ-CEHC and α-LA, and 4–5-fold for HVA and 5-HIAA. One of the reasons for this is that the background noise was significantly lower during the analysis for the PA-derivatives than for the HP-derivatives. These results demonstrate that PA is a more suitable reagent than HP for the sensitive detection of carboxylic acids.

3.3. Application of PA-derivatization to analysis of carboxylic acids in saliva

Saliva has recently been attracting attention as a new tool in clinical examinations and therapeutic drug monitoring [16,17]. The prime advantage of saliva is that it offers easy, noninvasive, stress-free and real-time repeated sampling where blood collection is either undesirable or difficult. No special training or equipment is needed and subjects can conveniently collect the samples themselves, if required. Thus, it is expected that saliva-based testing will be highly beneficial to the subjects (patients to be examined). However, a major disadvantage of using saliva is the low analyte concentration; a highly sensitive detection technique is needed for saliva testing. Based on this background information, the PA-derivatization, which was more effective than the HP-derivatization in increasing the ESI-MS/MS responses of carboxylic acids (Table 2), was applied to the trace detection of bile acids, γ-CEHC and HVA in saliva.

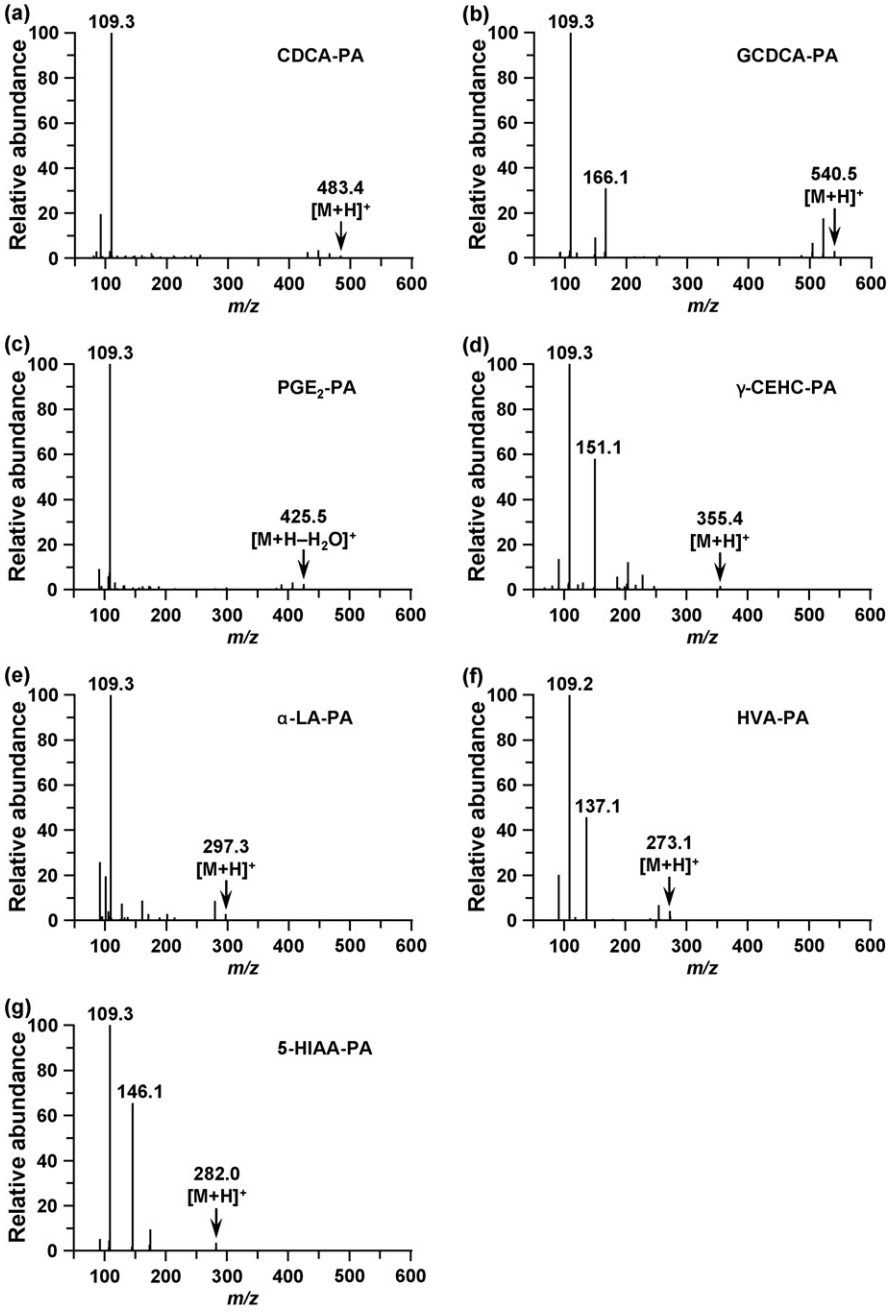

Fig. 4. ESI-MS/MS spectra of PA-derivatives of (a) CDCA, (b) GCDCA, (c) PGE_2, (d) γ-CEHC, (e) α-LA, (f) HVA and (g) 5-HIAA. The MS/MS spectra were recorded by the collisional activation of $[M+H]^+$ of the respective derivatives, except for PGE_2-PA ($[M+H-H_2O]^+$). The other conditions are described in Section 2.2 and Table 1.

3.3.1. Separation and detection of primary non-amidated bile acids in saliva

Analysis of bile acids, especially primary bile acids (CDCA, CA and their conjugates), is useful in the evaluation of hepatobiliary functions and in the diagnosis of related diseases, such as hepatitis, liver cancer, alcoholic liver injury and obstructive jaundice. To demonstrate the utility of the PA-derivatization, the analysis of CDCA and CA (primary non-amidated bile acids) in saliva was performed.

The saliva samples were collected from male subjects 2 and 6 h after meals and analyzed. Typical chromatograms are shown in Fig. 5, in which the peaks corresponding to the derivatized CDCA (t_R 4.3 min) and CA (t_R 3.1 min) were clearly detected with satisfactory shapes. The peak area ratios of CDCA and CA to D_4-CDCA (CDCA/D_4-CDCA and CA/D_4-CDCA) were larger in the sample collected 2 h after a meal (Fig. 5a). This result consistent with the fact that bile acid production/secretion increases after meals. Thus, by using LC–MS/MS combined with the PA-derivatization, the change in the bile acid production/secretion can be observed from the saliva analysis.

3.3.2. Separation and detection of γ-CEHC in saliva

Vitamin E functions as a lipophilic chain-breaking antioxidant that prevents lipid peroxidation [18]. γ-Tocopherol, one of the major forms of vitamin E, is often taken as a food supplement in hopes of not only the antioxidant effect, but also the diuretic effect (amelioration of swelling). However, the diuretic effect is not due to γ-tocopherol itself; γ-CEHC, a major metabolite of γ-tocopherol, is a true natriuretic factor [19]. For these reasons, we attempted the detection of γ-CEHC in saliva using the PA-derivatization followed by LC–ESI-MS/MS.

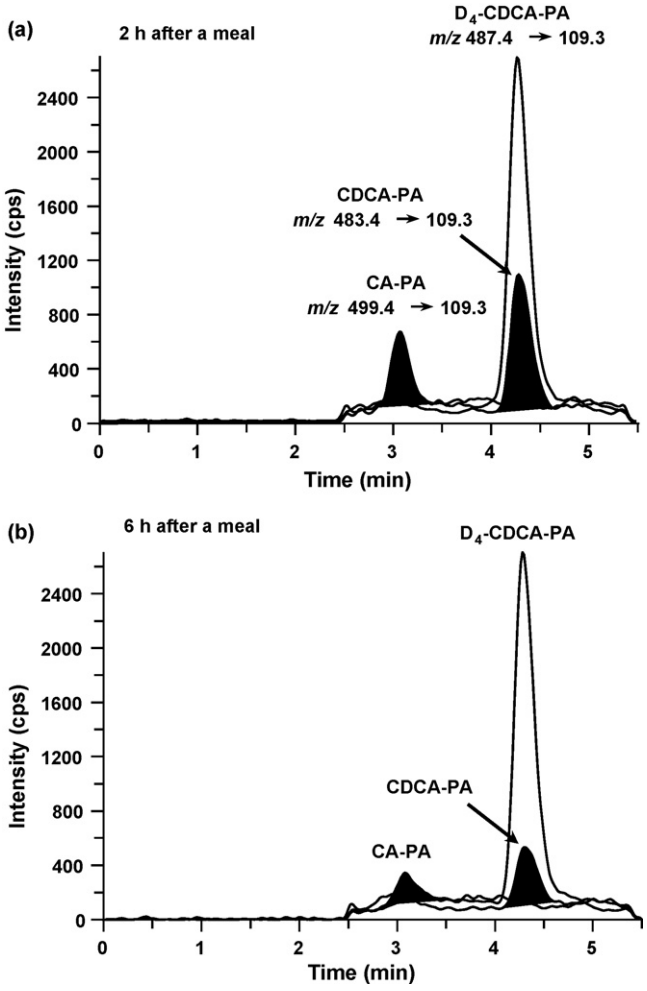

Fig. 5. Chromatograms of PA-derivatized CDCA and CA in saliva. Saliva samples obtained from a male healthy subject were spiked with D_4-CDCA and analyzed with the PA-derivatization. Saliva samples were collected (a) 2 h after a meal (collection time, 14:00) and (b) 6 h after a meal (collection time, 18:00). The LC–MS/MS conditions are described in Section 2.2 and Tables 1 and 2, but methanol–water (3:1, v/v) containing 0.1% (v/v) formic acid instead of methanol–water (4:1, v/v) containing 0.1% (v/v) formic acid was used as the mobile phase.

Two hundred microliters of saliva from the subjects, who had taken the γ-tocopherol supplement (67 mg) 6 h before the saliva collection, was pretreated as described in Section 2.7.2. A typical chromatogram is shown in Fig. 6a, in which the peak corresponding to the derivatized γ-CEHC was clearly observed at 3.7 min. On the contrary, when the same sample was analyzed without any derivatization, the peak corresponding to γ-CEHC (t_R 3.7 min) was almost at the noise level (Fig. 6b). Fig. 6c represents the chromatogram obtained by analyzing the saliva of a subject without the γ-tocopherol supplementation. This sample was subjected to the PA-derivatization followed by LC–MS/MS analysis. There was no interfering peak derived from the endogenous components and the derivatization reagents at the elution position of the derivatized γ-CEHC.

The PA-derivative of γ-CEHC provided a product ion at m/z 151.1 (Fig. 4d), which may be derived from the cleavage of the chroman moiety, together with the PA-derived product ion (m/z 109.3). The ratio of abundance of these ions (m/z 109.3/151.1) was also determined to confirm the purity of the peak corresponding to γ-CEHC-PA (t_R 3.7 min) in the saliva sample. The ratio of three different saliva samples [1.39 ± 0.01, mean ± standard deviation (S.D.)] was completely identical to that of the standard sample (1.39 ± 0.02, mean ± S.D. of triplicate measurements). This is the first reported instance for the detection of γ-CEHC in saliva of a subject having taken γ-tocopherol. These data indicate that the present method is highly sensitive and has enough specificity and practicality for biological sample analysis.

3.3.3. Separation and detection of HVA in saliva

The plasma HVA level is frequently measured as an indicator of central dopaminergic activity. A previous study found a significant correlation between the plasma and salivary HVA concentrations and suggested that the salivary HVA measurement can be a non-invasive alternative to the plasma measurement [20]. HPLC with electrochemical detection was used for the analysis of the salivary HVA, but the reported methods require a relatively large sample volume (0.5 ml) [20,21] and a long chromatographic run time (over 30 min) [21]. As shown in Fig. 7a, our method using the PA-derivatization followed by LC–ESI-MS/MS enabled the detection of HVA (t_R 5.5 min) with 0.1 ml of saliva and a relatively short chromatographic run time (9 min). Reducing the sample volume is an important consideration in clinical examinations; judging from the chromatogram in Fig. 7a, the sample volume could be further reduced in our assay. HMPP spiked in a saliva sample was

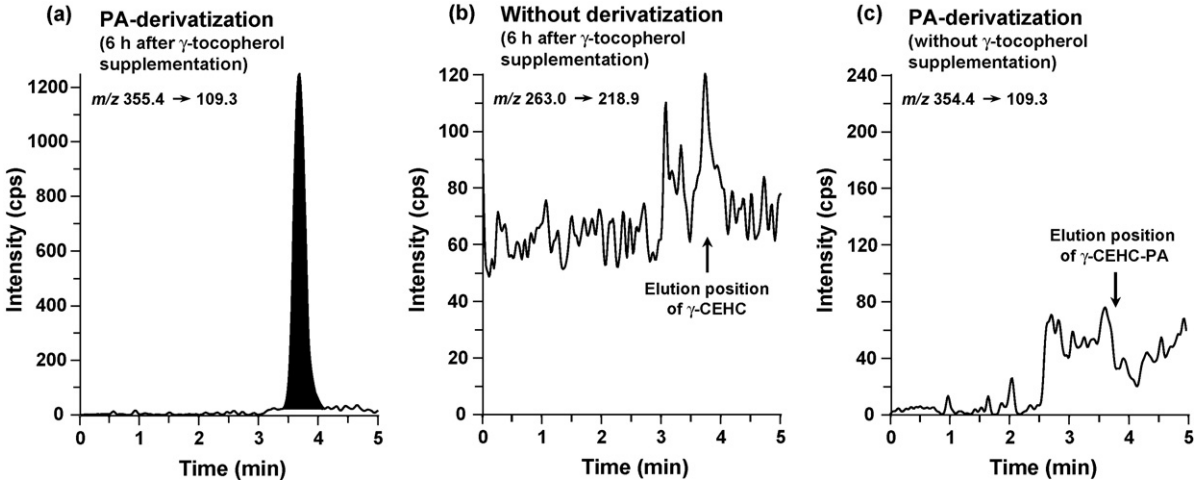

Fig. 6. Chromatograms of PA-derivatized and intact γ-CEHC in saliva. (a) A male healthy subject's saliva 6 h after the γ-tocopherol supplementation was analyzed with the PA-derivatization, (b) the same saliva was analyzed without any derivatization and (c) a male healthy subject's saliva without the γ-tocopherol supplementation was analyzed with the PA-derivatization. The LC–MS/MS conditions are described in Section 2.2 and Tables 1 and 2.

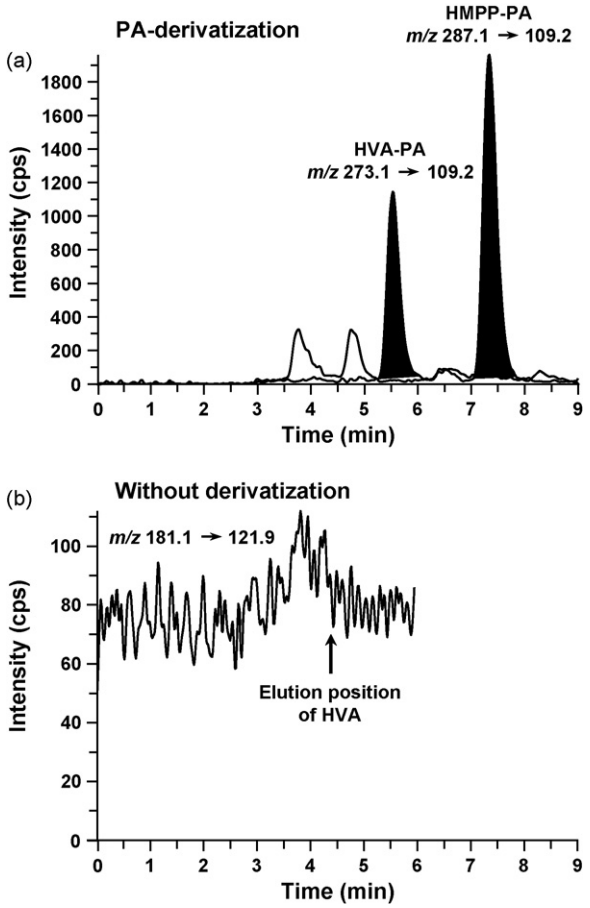

Fig. 7. Chromatograms of PA-derivatized and intact HVA in saliva. (a) A male healthy subject's saliva spiked with HMPP (IS) was analyzed with the PA-derivatization and (b) the same saliva (without spiking HMPP) was analyzed without any derivatization. The LC–MS/MS conditions are described in Section 2.2 and Tables 1 and 2, but methanol–10 mM ammonium formate (3:7, v/v) instead of methanol–10 mM ammonium formate (7:13, v/v) was used as the mobile phase in the analysis of HVA as the PA-derivative.

simultaneously analyzed (m/z 287.1 $[M+H]^+ \rightarrow$ 109.2, t_R 7.3 min), because it will be used as the internal standard (IS) in future quantitative assays. The intra-assay reproducibility (relative standard deviation, $n = 5$) of the determination of the HVA/HMPP ratio of a healthy subject's saliva was 3.6%. On the contrary, the peak corresponding to HVA was not detected at all without derivatization (Fig. 7b).

HVA-PA produced the intense product ion at m/z 137.1 [loss of ($C_5H_4NCH_2NHCO$+H) from the protonated molecule] together with the PA-derived product ion at m/z 109.2 (Fig. 4f). Using the ratio of abundance of these ions (m/z 109.2/137.1), the purity of the peak at 5.5 min in the saliva sample was examined. The ratio of three different saliva samples (2.14 ± 0.03, mean ± S.D.) and the standard sample (2.14 ± 0.04, mean ± S.D. of triplicate measurements) agreed with each other. These data proved that LC–MS/MS combined with the PA-derivatization is very valuable for the analysis of the salivary HVA.

4. Conclusion

We developed a simple and practical derivatization procedure for the enhanced detection of carboxylic acids using LC–ESI-MS/MS. The detection responses of the PA-derivatives were increased by 1–2 orders of magnitude over the intact carboxylic acids and the LODs were in the low femtomole range. The developed procedure has a sufficient applicability for the biological sample analysis; the PA-derivatization followed by LC–ESI-MS/MS enabled the detection of trace amounts of bile acids, γ-CEHC and HVA in saliva with a simple pretreatment, small sample volume and short analysis time. We are now studying the application of the present method for the qualitative and quantitative analyses of various physiologically active carboxylic acids, including drugs, in biological fluids and tissues.

Acknowledgements

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