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Determination of green tea catechins in human plasma using liquid chromatography–electrospray ionization mass spectrometry

Yoshinori Masukawa *, Yuji Matsui, Namii Shimizu, Naoki Kondou, Hidenori Endou, Michiya Kuzukawa, Tadashi Hase

Tochigi Research Laboratories, Kao Corporation, 2606 Akabane, Ichikai-machi, Haga, Tochigi 321-3497, Japan

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Abstract

A method for the sensitive and specific determination of eight green tea catechins, consisting of catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), catechin-3-gallate (CG), epicatechin-3-gallate (ECG), gallocatechin-3-gallate (GCG) and epigallocatechin-3-gallate (EGCG), in human plasma was established. For optimization of conditions for LC–ESIMS, the separation of the eight catechins was achieved chromatographically using Inertsil ODS-2 column combined with a gradient elution system of 0.1 M aqueous acetic acid and 0.1 M acetic acid in acetonitrile. Detection using a mass spectrometer was performed with selected ion monitoring at $m/z = 289$ for E and EC, 305 for GC and EGC, 441 for CG and ECG, and 457 for GCG and EGCG under negative ESI. A preparative procedure, consisting of the addition of perchloric acid and acetonitrile to the plasma for deproteinizing and the subsequent addition of potassium carbonate solution to remove excess acid, was developed. In six different plasma with the eight catechins spiked at two different concentrations, the average recoveries were in the range between 72.7 and 84.1%, which resulted from the matrix effect and preparative loss, with coefficients of variance being 8.2–19.8% among individuals. The levels of the catechins in prepared plasma solutions that were kept at 5 °C within 24 h were stable, which allows us to simply analyze many prepared plasma solutions using an autosampler overnight. When using this method to analyze the eight catechins in human plasma after oral ingestion of a commercial green tea beverage, we detected all the catechins absorbed into human blood for the first time. This also suggested that extremely small amounts of the eight catechins orally ingested may be absorbed based on each absorptive property for the catechins. The method should enable pharmacokinetic studies of green tea catechins in humans.

Keywords: LC–ESIMS; Green tea; Catechins; Deproteinizing; Human plasma

1. Introduction

Green tea catechins, which can be easily infused by hot water, have been extensively reported to possess various biological and pharmacological effects, such as anti-carcinogenic activities [1], anti-oxidant activities [2], lowering of plasma lipid [3] and glucose levels [4], and reducing obesity [5,6]. Among these, the anti-obesity effect in humans following oral ingestion of greater amounts of green tea catechins [6] has been recently reported, while obesity associated with many health risks (so-called lifestyle-related diseases) is a growing problem in many countries worldwide. Naturally occurring green tea catechins in an infusion of green tea leaves with hot water, which are widely consumed in Asian countries, consist of epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epicatechin (EC). On the other hand, Japanese have recently begun to prefer drinking a commercial canned or bottled green tea beverage, which has a different composition of catechins from the hot water infusion. Thus, owing to the epimerization of the four epi-catechins by heat treatment used for retort pasteurization in the manufacturing, four non-epi-forms, gallocatechin-3-gallate (GCG), catehin-3-gallate (CG), gallocatechin (GC) and catechin (C), are produced in the beverage [7,8]. Therefore, attention has been recently paid to the effects of these non-epi-forms on lipid metabolism [9].

To understand the pharmacokinetics of green tea catechins after oral ingestion of a green tea beverage, all eight green tea catechins must be determined in plasma. Among many methods

* Corresponding author. Tel.: +81 285 68 7416; fax: +81 285 68 7418.
E-mail address: masukawa.yoshinori@kao.co.jp (Y. Masukawa).

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for the analysis of green tea catechins [10,11], LC connected to an ultraviolet detector (UVD), a fluorescence detector (FLD), a chemiluminescence detector (CLD) and an electrochemical detector (ECD) have been used to determine the catechins in plasma [12–22]. However, these methods using LC did not determine all eight catechins in the plasma, due to their problems in sensitivity and specificity. While the use of sensitive and specific LC–MS for the analysis of the catechins has been recently increasing [23–25], LC–MS has not been applied to such analyses of actual plasma after ingestion, although it has been used for the analyses in model plasma with a few spiked catechins [26,27]. In addition, there are no preparative procedures that can be used for high-throughput analyses with simplicity and practicability, although high throughput performance for the analyses of green tea catechins in many plasma samples obtained from different volunteers at several periods after the ingestion is required for understanding the behaviors of the eight catechins absorbed into human blood among individuals and among populations. Thus, complex procedures including liquid–liquid extraction [12,17,21,22,26] and solid phase extraction [13,20] are troublesome and time-consuming. Other simple procedures, such as the addition of either of an acid or an organic solvent [14,15,18,19] and on-line extraction [27], do not permit us to allow the analysis of many plasma samples (intact plasma or prepared plasma solutions) with an autosampler, even at low temperature, due to the instability of the catechins in such non-prepared or prepared plasma samples.

The aim of this study was to establish a method for the sensitive and specific determination of eight green tea catechins in human plasma after oral ingestion of a green tea beverage. We have optimized the LC–ESIMS (liquid chromatography coupled to electrospray ionization mass spectrometry) conditions to simultaneously determine all eight catechins and we have developed a simple preparative procedure for deproteinizing plasma, in which the catechins are stable at 5 °C within 24 h after preparation. This method has been applied to analyze the eight catechins in the plasma after the ingestion of a commercial green tea beverage, in which we succeeded for the first time in detecting and determining all eight catechins in human plasma.

2. Experimental

2.1. Chemicals

Authentic green tea catechins such as C, EC, GC, EGC, CG, ECG, GCG and EGCG were purchased from Kurita Industrial (Tokyo, Japan). Ultra-pure water was prepared using a Milli-Q purification system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade or of HPLC grade. A commercial green tea beverage, which has been approved as a food for specified health use in the Japanese market by the Ministry of Health, Labour and Welfare in Japan, was orally ingested. This beverage contained 1.66 mg/ml total green tea catechins, consisting of 0.12 mg/ml C, 0.08 mg/ml EC, 0.37 mg/ml GC, 0.22 mg/ml EGC, 0.11 mg/ml CG, 0.09 mg/ml ECG, 0.36 mg/ml GCG and 0.31 mg/ml EGCG, as determined by quantitative HPLC with UV detection at 280 nm.

Each stock standard solution (ca. 1 mg/ml) was prepared by dissolving a known amount of each authentic compound in buffer consisting of 0.1 M sodium phosphate, 25 vol% methanol, 0.5 wt% ascorbic acid and 0.025 wt% disodium ethylenendiaminetetraacetate (pH 3.9) and their small aliquots were stored at −80 °C until used. The stock solutions remained stable under −80 °C for at least 6 months. Working authentic mixtures were always obtained just before use by further dilutions of the stock solution aliquots with the buffer.

2.2. Apparatus and analytical conditions

An Agilent 1100 Series LC-MSD SL system (single quadrupole) equipped with ChemStation software, an 1100 well plate autosampler, a diode array detector (Agilent Technologies, Palo Alto, CA, USA) and an Inertsil ODS-2 column 2.1 mm Ø × 250 mm (GL Science, Tokyo, Japan) was used for the analysis of the eight catechins. In this LC-MSD system, an 1100 binary pump connected to eluents A (0.1 M aqueous acetic acid) and B (0.1 M acetic acid in acetonitrile) was used. The mobile phases were consecutively programmed as follows: an isocratic elution of A 94% (B 6%) for 5 min, a linear gradient of A 94–90% between 5 and 10 min, a linear gradient of A 90–80% between 10 and 20 min, a linear gradient of A 80–77% between 20 and 29 min, an isocratic elution of A 77% for 6 min, a linear gradient of A 77–0% between 35 and 40 min, and finally an isocratic elution of A 94% from 40.1 to 65 min (a total run time of 65 min). The injection volume was 10 μl of each sample solution. The column temperature was maintained at 35 °C and was eluted at flow rate of 0.2 ml/min. Electrospray ionization (ESI) in the mass spectrometer was performed with the following parameters: ionization; negative ion mode, V-cap voltage; 3000 V, fragmentor voltage; 140 V, flow of heated dry nitrogen gas; 10 l/min, heater temperature of gas; 350 °C, and nebulizer gas pressure; 50 psi. Negative ions in scan measurements were acquired from m/z = 100–1000 with a scan time of 0.67 s per cycle. The mass spectrometer was also operated in selected ion monitoring (SIM) with m/z = 289 for E and EC at 15–37 min in the run time, 305 for GC and EGC at 15–31 min, 441 for CG and ECG at 37–50 min, and 457 for GCG and EGCG at 31–50 min. When parameters associated with an impact on performance of ESI were examined, intensities of the monitoring ions for the eight catechins were most strongly affected by the fragmentor voltage of all parameters tested. Based on the test of the fragmentor voltages in the range between 40 and 240 V, 140 V providing with higher intensities for the eight catechins was chosen. Each calibration line was generated by plotting the concentrations of an authentic compound against their peak areas.

2.3. Human plasma

The protocol was approved by the Ethical Committee of the Kao Corporation of Japan, based on the Recommendations from the Declaration of Helsinki. All plasma samples were obtained from six healthy nonsmoking male volunteers (26–38 years, 55–70 kg body weight, #1 to #6), who had not consumed tea or tea-related beverages for 3 days prior to this experiment. After

fasting for 14 h, 350 ml of the beverage containing 581 mg green tea catechins was orally ingested and no other drinks were taken except water. Their blood samples were taken using heparin as an anticoagulant before and 1, 2, 3, 4, 6 and 8 h after the ingestion, and were immediately centrifuged at 4000 × *g* for 10 min at 5 °C to prepare their plasma. One milliliter of plasma was mixed with 20 μl 0.4 M phosphate buffer (pH 3.6) containing 20% ascorbic acid and 0.1% disodium ethylenendiaminetetraacetate in a microtube and was stored at −80 °C until analysis for up to 3 months. After the storage at −80 °C for 3 months, more than 92% of the initial amounts in the blank plasma with the eight spiked catechins were recovered.

2.4. Sample preparation

To 250 μl of the plasma thawed just prior to use, 25 μl 6 M perchloric acid and 125 μl acetonitrile were added. The mixture was vigorously mixed for 2 min with a vortex mixer in a polypropylene tube. The mixture was kept at 5 °C for 30 min, and was then centrifuged at 20,000 × *g* for 5 min at 5 °C in a Centrifuge 5417R (Eppendorf, Hamburg, Germany). The supernatant and 100 μl 0.75 M potassium carbonate solution were combined moderately for 30 s. Insoluble potassium perchlorate in the tube was precipitated by centrifugation at 20,000 × *g* for 5 min at 5 °C. The resulting supernatants were subjected to LC–ESIMS.

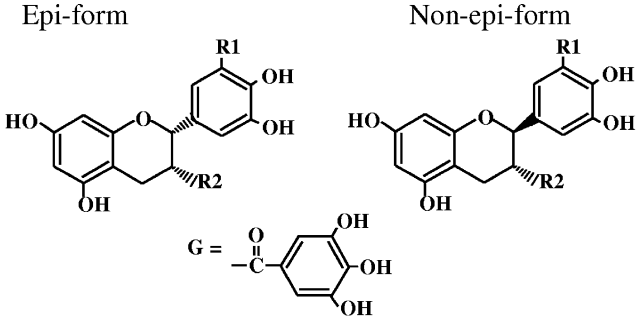
2.5. Preparation of plasma solution spiked

In the preparation of plasma solutions spiked with eight authentic green tea catrechins, six different blank plasma obtained from six male volunteers (#1 to #6) before oral ingestion of a green tea beverage were used. To estimate matrix effect [28], the blank plasma was prepared according the procedure described in Section 2.4, followed by the addition of known amounts of the authentic catechins to the plasma solutions to finally get desired concentrations. Thus, 90 vol of the prepared plasma solution was transferred into a polypropylene tube and 10 vol of the authentic solution was then added into. In contrast, to estimate recovery (= process efficiency [28]), the blank plasma was prepared according to the above method except for the addition of authentic catechins before the preparative procedure.

3. Results

3.1. LC–ESIMS conditions

The LC separation of the eight authentic catechins was examined using two mobile phases of 0.1 M aqueous acetic acid and 0.1 M acetic acid in acetonitrile. Acetic acid was chosen as an additive rather than trifluoroacetic acid or formic acid since it provides the most abundant deprotonated molecular ions $[M-H]^-$ characteristic for green tea catechins [29] under negative ESI. An appropriate gradient elution with the two mobile phases as described in Section 2.2 enabled us to mutually separate the eight catechins including the four non-epi-forms and the four epi-forms (Fig. 1) within 40 min. Fig. 2 shows a representative LC–ESIMS total ion chromatogram of the eight catechins, which elute in the order of GC, EGC, C, EC, EGCG, GCG, ECG and CG, under the optimized LC conditions. An isomer pair of a non-epi-form and an epi-form with gallate had a different chromatographic elution from that without gallate. Thus, the epi-forms with gallate (EGCG and ECG) eluted prior to the non-epi-forms with gallate (GCG and CG), while the non-epi-forms without gallate (GC or C) eluted prior to the epi-forms without gallate (EGC and EC). This chromatographic behavior is in close agreement with previous reports that analyzed



Analyte		R1	R2	Monitoring ion
Epi-form	Epicatechin	H	OH	$[M-H]^-$: m/z = 289
	Epicatechin gallate	H	OG	$[M-H]^-$: m/z = 441
	Epigallocatechin	OH	OH	$[M-H]^-$: m/z = 305
	Epigallocatechin gallate	OH	OG	$[M-H]^-$: m/z = 457
Non-epi-form	Catechin	H	OH	$[M-H]^-$: m/z = 289
	Catechin gallate	H	OG	$[M-H]^-$: m/z = 441
	Gallocatechin	OH	OH	$[M-H]^-$: m/z = 305
	Gallocatechin gallate	OH	OG	$[M-H]^-$: m/z = 457

Fig. 1. Chemical structures of eight green tea catechins.

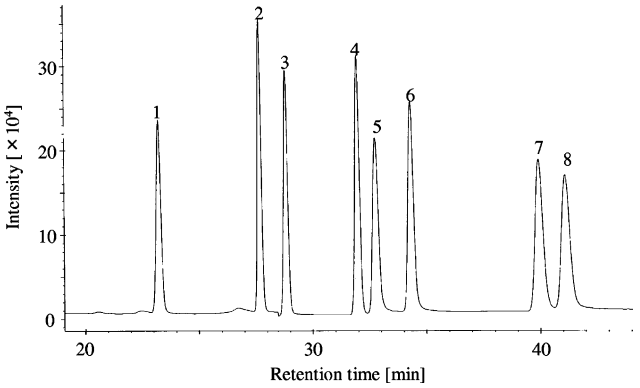


Fig. 2. LC–ESIMS total ion chromatogram of eight green tea catechins in an authentic solution (each 100 ng/ml). Peaks: 1 = GC, 2 = EGC, 3 = C, 4 = EC, 5 = EGCG, 6 = GCG, 7 = ECG, 8 = CG. LC conditions: column, Inertsil ODS-2 (2.1 mm Ø × 250 mm); column temperature, 35 °C; mobile phase, (A) 1 M aqueous acetic acid, and (B) 0.1 M acetic acid in acetonitrile; flow rate, 0.2 ml/min; gradient elution, see Section 2; injection, 10 μl. ESIMS conditions: ionization, negative mode; V-cap voltage, 3000 V; fragmentor voltage, 200 V; flow of heated nitrogen gas, 10 l/min; heated temperature of gas, 350 °C; nebulizer gas pressure, 50 psi; scan range, m/z = 100–1000; scan time, 0.67 s per cycle.

green tea catechins using reversed phase LC [20,22]. This may be attributable to differences in the molecular hydrophobicity between a non-epi-form and the corresponding epi-form according to the presence or absence of gallate, as GCG, CG, EGC and EC are more hydrophobic than EGCG, ECG, GC and C, respectively, based on their molecular configurations. In ESIMS spectra of the eight catechins under optimized conditions of LC, the most abundant deprotonated molecular ions, $[M-H]^-$ m/z = 289, 305, 441 and 457, were observed in both C and EC, both GC and EGC, both CG and ECG, and both GCG and EGCG, respectively, and these ions were selected as targets for SIM in this analysis.

Reproducibility (C.V.%, n = 6), limit of detection (LOD, S/N = 3) and limit of quantification (LOQ, S/N = 10) for the eight authentic catechins under optimized conditions of LC–ESIMS were obtained (Table 1). The C.V.% for retention times and peak areas of six consecutive analyses of a 10 ng/ml solution for each of the catechins were in the range of 0.03–0.09 and 1.5–3.7%, respectively. The LOD and LOQ for 10 μl injection of the authentic solution ranged from 0.19 to 0.25 ng/ml and from 0.63 to 0.83, respectively. As for the calibration lines, all slopes for the authentic catechins were reproducibly constructed as seen in Table 2, which showed C.V.% = 1.0–1.6, with the correlation coefficients being greater than 0.9985 in the range of 1–100 ng/ml. These results indicate that this optimized LC–ESIMS technique provides sufficient sensitivity and precision for the analysis of the eight catechins.

Table 1
Reproducibility (R.S.D. in %), limit of detection (LOD), and limit of quantification (LOQ) for authentic eight green tea catechins

Green tea catechins	Reproducibility (R.S.D.%)[a]		LOD[b] (ng/ml)	LOQ[c] (ng/ml)
	Retention time	Area		
GC	0.09	2.2	0.19	0.63
EGC	0.05	2.0	0.25	0.83
C	0.04	1.5	0.22	0.73
EC	0.04	1.5	0.21	0.69
EGCG	0.04	3.2	0.25	0.82
GCG	0.04	2.7	0.19	0.64
ECG	0.03	2.6	0.25	0.63
CG	0.03	3.7	0.21	0.70

[a] Six analyses of a 10 ng/ml solution for each of the catechins were consecutively performed.
[b] Defined as S/N = 3 for 10 μl injection.
[c] Defined as S/N = 10 for 10 μl injection.

3.2. Preparative procedure for deproteinizing plasma

A simple preparative procedure for deproteinizing plasma by the addition of an acid and an organic solvent was examined using blank plasma spiked with the eight authentic catechins at a concentration of 40 ng/ml each before a preparative procedure. When each of the three acids chosen (trichloroacetic acid, trifluoroacetic acid and perchloric acid) was added to the plasma, all acids added beyond 0.3 M in the plasma precipitated the proteins after centrifugation. Among them, the addition of perchloric acid to the plasma was superior to trichloroacetic acid or trifluoroacetic acid, due to the relatively well shaped-peaks of the catechins on LC–ESIMS chromatograms. Meanwhile, when acetonitrile was chosen as an organic solvent [18], the addition of 25% acetonitrile in the plasma solution (v/v) was optimal, because less than 20% acetonitrile caused lower recoveries of the spiked catechins while more than 30% acetonitrile resulted in deteriorated peak shapes of GC and EGC which eluted earlier on this LC–ESIMS chromatogram for 10 μl injection (data not shown). Based on these examinations, the addition of 25 μl 6 M perchloric acid and 90 μl acetonitrile to 250 μl of the plasma was tentatively chosen for optimal deproteinization of the plasma. Furthermore, a procedure for the removal of excess perchloric acid was examined since significant tailing of the peaks were often observed on the chromatograms, which was probably due to the strong acidity of the deproteinized plasma solution. According to a report in which a supernatant of serum acidified by perchloric acid was neutralized with potassium carbonate solution [30], 100 μl 0.75 M potassium carbonate solution (corresponding to the equivalent moles of potassium to those of perchlorate originating from 25 μl of 6 M perchloric acid) was added and insoluble salts (potassium perchlorate) were removed by centrifugation. Consequently, the preparative procedure as described in Section 2.4 was established to deproteinize plasma. Using this procedure, blank plasma and that with the eight catechins spiked at the concentration of 1 ng/ml each before the preparative procedure were analyzed. In the blank plasma, there are almost no peaks in all LC–ESIMS SIM chromatograms except peaks that elutes at ca. 24–25 min in the chromatograms of m/z = 289 and 307 being different retention times from the catechins (Fig. 3a). On the other hand, well shaped-peaks for each of the eight catechins were resolved on the LC–ESIMS SIM chromatograms of the spiked blank plasma (Fig. 3b). Furthermore, the stability of the eight catechins in the prepared plasma solutions was examined by consecutive analyses of blank plasma containing the eight catechins spiked at a concentration of 40 ng/ml each, which were processed by the

Table 2
Slope, intercept and correlation coefficient of calibration lines for authentic solutions ($n = 6$) and six different plasma solutions (#1 to #6) spiked after preparative procedure

Green tea catechins	Mean ± S.D. (coefficient of variance in %)		
	Slope[a]	Intercept[a]	Correlation coefficient
Authentic solution[b]			
GC	14456 ± 180 (1.2)	−3434 ± 366 (10.7)	0.9990 ± 0.0001 (0.01)
EGC	14788 ± 234 (1.6)	−3671 ± 440 (12.0)	0.9990 ± 0.0002 (0.02)
C	15853 ± 159 (1.0)	−2322 ± 371 (16.0)	0.9997 ± 0.0001 (0.01)
EC	15433 ± 177 (1.1)	−2081 ± 324 (15.6)	0.9997 ± 0.0001 (0.01)
EGCG	11308 ± 139 (1.2)	−2209 ± 209 (9.5)	0.9988 ± 0.0004 (0.04)
GCG	11476 ± 150 (1.3)	−1593 ± 634 (39.8)	0.9987 ± 0.0003 (0.03)
ECG	15426 ± 177 (1.1)	−169 ± 699 (414)	0.9985 ± 0.0002 (0.02)
CG	16896 ± 187 (1.1)	−1542 ± 690 (44.7)	0.9990 ± 0.0002 (0.02)
Plasma solution spiked after preparative procedure[c]			
GC	11745 ± 1116 (9.5)	−617 ± 343 (55.6)	0.9999 ± 0.0001 (0.01)
EGC	11973 ± 2119 (17.7)	−1339 ± 358 (26.7)	0.9997 ± 0.0002 (0.02)
C	12349 ± 1605 (13.0)	−1466 ± 925 (63.1)	0.9998 ± 0.0002 (0.02)
EC	12117 ± 1648 (13.6)	−986 ± 959 (97.3)	0.9997 ± 0.0003 (0.03)
EGCG	10889 ± 1263 (11.6)	−1703 ± 674 (39.6)	0.9994 ± 0.0005 (0.05)
GCG	10450 ± 1463 (14.0)	−1660 ± 759 (45.7)	0.9994 ± 0.0005 (0.05)
ECG	13864 ± 1192 (8.6)	−2703 ± 1341 (49.6)	0.9996 ± 0.0003 (0.03)
CG	15041 ± 1790 (11.9)	−3691 ± 1731 (46.9)	0.9991 ± 0.0004 (0.04)

[a] Calculated from the equation $y = Ax + B$, where x is an injected concentration in ng/ml, y is a peak area, A is a slope, and B is an intercept.
[b] Six calibration lines were produced by the consecutive injections of six sets of authentic solutions in the range between each 1 and 100 ng/ml (1, 5, 10, 50 and 100 ng/ml).
[c] Six calibration lines were produced by the consecutive injections of six different plasma solutions spiked with the concentrations of each 1 to 100 ng/ml (1, 5, 10, 50 and 100 ng/ml) after the preparative procedure.

preparative procedure for deproteinization and then analyzed by LC–ESIMS. After storage at 5 °C for 24 h, more than 95% of the initial peak areas in the eight catechins were detected (data not shown).

Reproducibility (C.V.%, $n = 6$), limit of detection (LOD, S/N = 3), limit of quantification (LOQ, S/N = 10) (Table 3) and calibration lines (Table 2) for plasma solutions spiked after the optimized preparative procedure were obtained. The C.V.% for retention times and peak areas of six consecutive analyses of one blank plasma spiked at the concentration of 10 ng/ml were in the range of 0.02–0.04 and 3.2–5.9% for each of the catechins, respectively. The C.V.% for peak areas of the catechins in the blank plasma spiked was higher than that obtained from the authentic solutions but acceptable (Tables 1 and 3). The LOD and LOQ for 10 μl injection of the blank plasma spiked ranged from 0.25 to 0.32 ng/ml and from 0.75 to 1.06, respectively, which were slightly higher than those of the authentic solutions (Tables 1 and 3). The calibration lines were different from those of the authentic solutions in terms of lower absolute values and higher C.V.% of slopes for each of the catechins tested although each calibration line had a much higher linearity as presented in correlation coefficients and their C.V.% of Table 3. The lower values of the slope indicate the presence of matrix effect [28], by which the slope values are decreased to ca. 80% of those of the authentic solutions in the non-gallate forms and ca. 90% in the gallate forms, under this analytical condition. In addition, the higher C.V.% indicates that this matrix effect is variable dependent on plasma obtained from different individuals. On the other hand, overall recoveries from process efficiency [28] were obtained by analyzing the eight catechins spiked into blank plasma before the preparative procedure (Table 4). At two different concentrations, the average recoveries from process efficiency were in the range between 72.7 and 84.1% with C.V.% being 8.2–19.8% among six individuals. Data not shown, but the C.V.% of recoveries in the eight catechins within one plasma (#6, two concentrations spiked, $n = 6$ each) ranged from 2.6 to 5.6%. Since process efficiency is determined by both matrix effect and extraction efficiency [28], these results revealed that there are extraction losses that are roughly estimated to ca. 10% of the eight catechins under the this preparative procedure.

Table 3
Reproducibility (R.S.D. in %), limit of detection (LOD), and limit of quantification (LOQ) for one plasma solution (#1) spiked after preparative procedure

Green tea catechins	Reproducibility (R.S.D.%)[a]		LOD[b] (ng/ml)	LOQ[c] (ng/ml)
	Retention time	Area		
GC	0.04	4.0	0.27	0.89
EGC	0.02	5.9	0.25	0.75
C	0.02	4.0	0.26	0.86
EC	0.02	3.9	0.27	0.90
EGCG	0.02	3.9	0.26	0.86
GCG	0.02	3.2	0.27	0.88
ECG	0.02	3.9	0.30	0.97
CG	0.02	4.8	0.32	1.06

[a] Six analyses of a plasma solution spiked at 10 ng/ml for each of the catechins were consecutively performed.
[b] Defined as S/N = 3 for 10 μl injection.
[c] Defined as S/N = 10 for 10 μl injection.

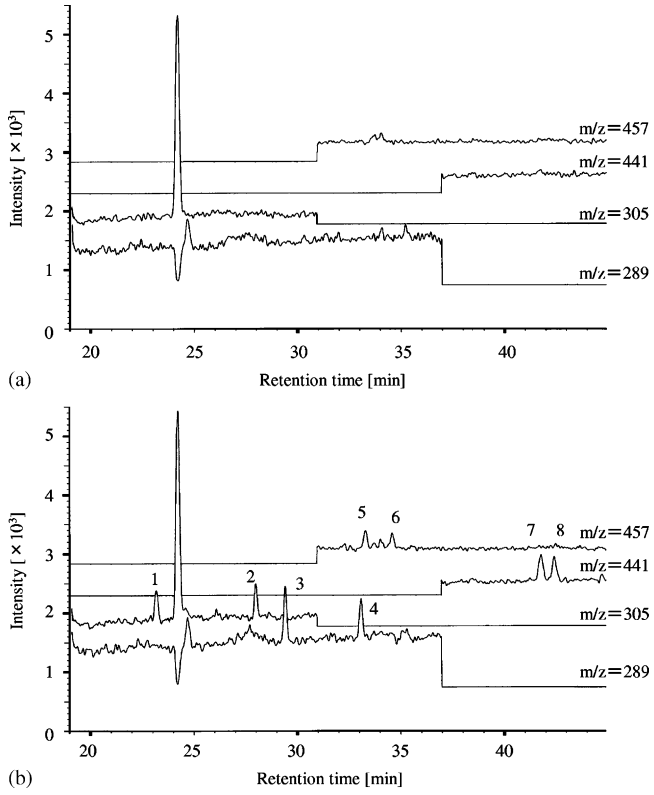


Fig. 3. LC–ESIMS SIM chromatogram in blank plasma (a) and that with eight authentic green tea catechins, each 1 ng/ml, spiked (b) after preparative procedures for deproteinizing. Peaks: see Fig. 2. LC–ESIMS conditions: see Fig. 2 except for fragmentor voltage 140 V and SIM used with $m/z = 289$ for E and EC, 305 for GC and EGC, 441 for CG and ECG, and 457 for GCG and EGCG. Procedures for deproteinizing: addition of 25 μl 6 M perchloric acid and 125 μl acetonitrile to 250 μl plasma were followed by the addition of 100 μl of 0.75 M potassium carbonate solution.

3.3. Analyses of green tea catechins in human plasma

The established method was applied to characterization of concentration versus time profiles for the eight catechins in human plasma after oral ingestion of 350 ml commercial green tea beverage containing total 581 mg of the catechins, using calibration lines of authentic solutions. Fig. 4 shows LC/ESIMS SIM chromatograms of the eight catechins in the plasma of one subject 2 h after the ingestion, in which each of the eight

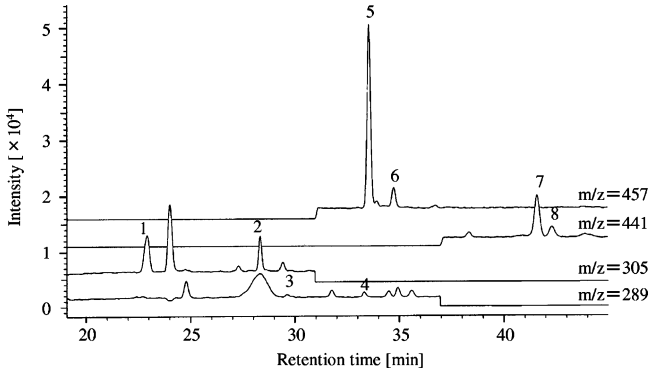


Fig. 4. LC–ESIMS SIM chromatograms of eight green tea catechins in human plasma of a subject 2 h after ingestion of 350 ml commercial green tea beverage. Peaks: see Fig. 2. LC–ESIMS conditions: see Fig. 3.

catechins was detected without any interference by co-existing components, despite the extremely smaller peaks for C and EC. The co-existing peaks, such as observed in the chromatograms of $m/z = 289$ and 307 except for larger peaks that elute at ca. 24–25 min (Fig. 4), seemed to result from the metabolites of the catechins because these peaks were not detected in the blank plasma (Fig. 3). The concentration versus time profiles for the eight catechins, obtained from all five subjects (#1 to #5), are depicted in Fig. 5. The level of each of the eight catechins increased after the ingestion, and reached maximum concentrations in the plasma at 1–2 h. In 1 h after the ingestion, the average concentration of EGCG in the plasma was the highest, followed by the order of ECG, GC, GCG, EGC, CG, EC and C. The pharmacokinetic AUC (area under the blood concentration time curve) that is a representative blood concentration was calculated for each of the eight caechins in the plasma (Table 5). First of all, the concentrations of the catechins in the plasma were remarkably lower than those in the beverage ingested. Table 5 also shows that the concentrations of GC and GCG were the highest in the beverage ingested while the concentration of EGCG was the extremely highest in the plasma, and that the beverage ingested contained almost the identical concentrations of C and ECG to those of CG and EC, respectively, while the plasma concentrations of CG and ECG were remarkably higher than C and EC, respectively.

Table 4
Overall recovery and coefficient of variance of eight green tea catechins spiked into human plasma

Green tea of catechins	Spiked concentration; 10 ng/ml[a]		Spiked concentration; 40 ng/ml[a]	
	Recovery (mean ± S.D.)	Coefficient of variance (%)	Recovery (mean ± S.D.) (%)	Coefficient variance (%)
GC	72.7 ± 8.8	18.2	76.0 ± 9.8	12.8
EGC	76.3 ± 15.1	19.8	80.7 ± 11.8	14.6
C	80.6 ± 11.3	14.0	76.5 ± 10.2	13.3
EC	78.6 ± 11.3	14.3	75.2 ± 9.7	12.9
EGCG	82.2 ± 9.8	11.9	84.1 ± 6.9	8.2
GCG	76.1 ± 13.2	17.3	83.8 ± 8.5	10.2
ECG	78.3 ± 14.1	18.0	81.2 ± 7.4	9.1
CG	76.6 ± 8.5	11.1	83.6 ± 10.1	12.0

[a] Six different plasma (#1 to #6) spiked before the preparative procedure was used (each $n = 1$).

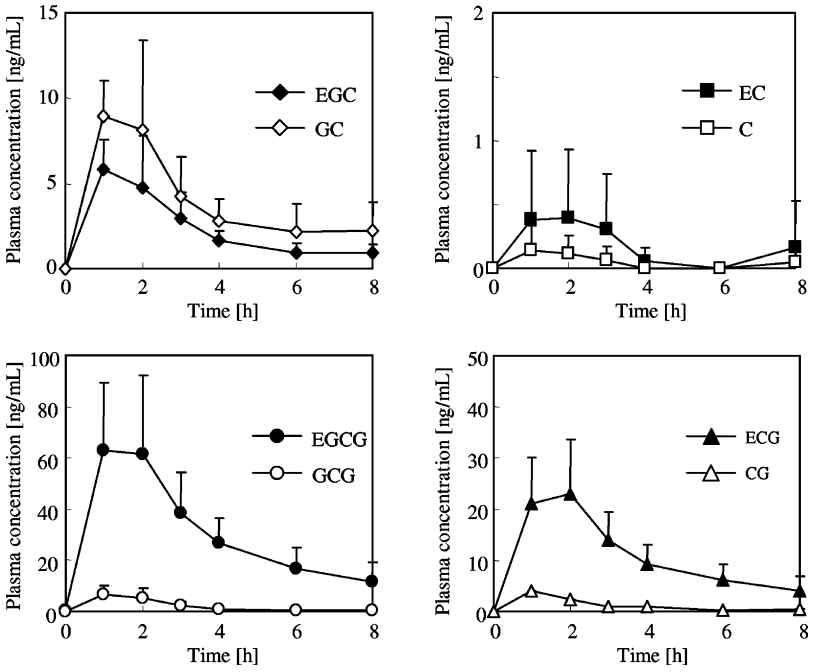


Fig. 5. Concentration–time profiles of eight green tea catechins in human plasma after ingestion of 350 ml commercial green tea beverage. Each point and vertical bar indicates the mean ± S.D. for five subjects (#1 to #5).

Table 5
Concentrations of eight green tea catechins in human plasma and green tea beverage ingested

Green tea catechin	Concentration	
	Plasma (ng h/ml)[a]	Green tea (mg/ml)
GC	35.5 ± 13.5	0.37
EGC	22.2 ± 5.5	0.22
C	<0.1	0.12
EC	0.3 ± 0.7	0.08
EGCG	261.2 ± 79.1	0.31
GCG	14.6 ± 9.6	0.36
ECG	83.4 ± 30.8	0.09
CG	9.0 ± 6.4	0.11

[a] Calculated based on the pharmacokinetic AUC. Mean ± S.D. were expressed.

4. Discussion

In this study, LC–ESIMS conditions were optimized for the sensitive and specific determination of eight green tea catechins. In the optimized LC–ESIMS, the chromatographic separation of the eight catechins is achieved by a reversed phase column Inertsil ODS-2 combined with a gradient elution system of two mobile phases. Mass spectrometric detection is performed with SIM at $m/z = [M - H]^-$ for each isomer pair of catechins under negative ESI. Since the LOD in our LC–ESIMS is 0.19–0.25 ng/ml (GC, EGC, C, EC, EGCG, GCG, ECG and CG) for 10 μl injection of an authentic solution, the sensitivity is estimated to be almost equivalent to the LC-ECD with a microbore column [22]. Although this technique used by Kotani et al. [22] seems to be one of the most sensitive methods of all [12–22] and can be used for the detection of eight authentic catechins in an authentic solution, Kotani et al. failed to detect all eight catechins in human plasma due to interference by the endogenous components. Compared with this, our method based on SIM of LC–ESIMS can be applied to specifically detect all the eight catechins in human plasma without any interference, as shown in Fig. 4. By optimizing LC–ESIMS conditions with higher sensitivity and specificity, we succeeded in the simultaneous detection of all eight catechins in the plasma.

Although at the early stage of this study we had used a simple preparative procedure based on the addition of only organic solvents to the plasma for deproteinizing, we stopped using them and decided to develop a new and more effective preparative procedure. This was because preparation of plasma solutions by its simple procedure using organic solvents always had to be performed just prior to the LC–ESIMS analysis, due to the instability of the eight catechins in the solutions. The newly developed preparative procedure is generated by the addition of perchloric acid and acetonitrile to precipitate the proteins, and the subsequent addition of potassium carbonate solution to neutralize excess perchloric acid. When matrix effect and process efficiency [28] (= overall recovery involving both the preparative procedure and the subsequent LC–ESIMS) in this method were evaluated, we found the presence of significant matrix effect, which is variable among the plasma obtained from different individuals but reproducible within those obtained from one individual. Due to this matrix effect influencing process efficiency, the measured quantitative values in this analysis seem to correspond to approximately 70–85% of actual ones with high variability among individuals, despite reproducibility being obtained for the plasma of each individual. Also, we can roughly estimate that there are extraction losses, almost 10% of the eight catechins present in the plasma, in this method. If we use an

optimal internal standard, which currently we cannot choose, or blank plasma solutions spiked with the authentic catechins after the preparative procedure for a calibration, more accurate quantitative values may be acquired. However, they may not be helpful for the reduction in the variation of the matrix effect among the plasma obtained from different individuals. Therefore, in this study, we have adopted a simple calibration method using the authentic solutions. Only, when using this method, it should be noted that measured quantitative values are not absolute and can be roughly used as relative ones, based on that they correspond to approximately 70–85% of actual ones with the variation among the plasma obtained from different individuals. A more effective method for determination of the catechins in human plasma with higher accuracy and lower individual variation must be explored in near future.

To analyze green tea catechins in the plasma, repeated liquid–liquid extraction with organic solvents [12,17,21,22,26], solid phase extraction [13,20], the addition of an acid or an organic solvent [14,15,18,19] and on-line extraction [27] were conventionally used to remove the plasma proteins. Our procedure is characterized by its simplicity, compared with repeated liquid–liquid extraction [12,17,21,22,26] or solid phase extraction [13,20]. Although there was a possibility that the strong acid, perchloric acid used would influence the hydrolysis of conjugated-form catechins present together with free-forms in the plasma, additional experiments indicated that our proposed procedure induces little hydrolysis of the conjugated-forms. Thus, the measured levels of the catechins in the plasma including free-forms and conjugated-forms were not increased with the increasing incubation time after the addition of perchloric acid and acetonitrile, whereas the substantial and reproducible levels of the conjugated-forms were always observed when incubation with β-glucuronidase and sulfatase was applied (data not shown). Therefore, such quantitative values of the catechins as determined using our proposed procedure can be thought to be almost equivalent to the levels of the free-forms in the plasma. Furthermore, the stability of the catechins in the prepared plasma solutions at 5 °C within 24 h allows us to analyze many plasma solutions that were prepared and subsequently placed within an autosampler overnight. It should be noted that the preparative procedure established in this study is practically available in terms of its simplicity.

Using the optimal LC–ESIMS analysis combined with the preparative procedure for deproteinizing plasma, the eight catechins (GC, EGC, C, EC, EGCG, GCG, ECG and CG) in the plasma after oral ingestion of a commercial green tea beverage have been detected for the first time. Thus, to our knowledge, there are no previous papers on the detection of all eight green tea catechins in human or animal plasma although some methods can detect a few of them as follows: EGCG and EGC in human plasma [12]; only C in rabbit plasma [13]; only EGCG in rat plasma [14]; only EGCG in human serum [15]; only EGCG in rat and human plasma [16]; only EGCG in human plasma [17]; only C in human plasma [18]; C and EC in rat plasma [19]; EGC, EC, EGCG and ECG in human plasma [20]; EGC, EC and EGCG in human plasma [21]; GCG, EC, EGCG, ECG and CG in human plasma [22].

The phenomenon that maximum concentrations of the eight catechins in human plasma were observed 1–2 h after the ingestion, with EGCG being the highest, is in accord with previous papers [15,20–22] describing concentrations of green tea catechins absorbed into the plasma after the ingestion of green tea (infusion or beverage). Based on the comparison of the concentration for each of the eight catechins between the plasma (pharmacokinetic AUCs) and the beverage ingested, we can first understand that almost all catechins orally ingested may be excreted to the outside of human body but only extremely small amounts of the eight catechins may be absorbed into human blood. In addition, despite absolutely lower levels in the absorption into human blood, our results suggest that different absorption among the eight catechins may occur because the AUCs do not necessarily correspond to the concentrations of catechins in the beverage ingested. Although it is possible that this difference may be ascribed to each absorptive property such as EGCG and EGC being easily absorbed into human blood, we are now considering another possibility that metabolic alterations of the absorbed free forms into their conjugated and/or methylated forms [12,21–22,31,32] may occur. Because it is known that the level of EC in the plasma, which was extremely low in this study, is higher in the conjugated form than in the free form [21,22], and in rat plasma after oral ingestion of EC, complex metabolites consisting of glucuronide conjugates, sulfate ones, glucuronide/sulfate ones and methylated ones, were found [32]. Therefore, in the future, complete concentration versus time profiles for the eight catechins, including their free forms and complex metabolites, in the plasma after ingestion should be clarified to understand the difference in the catechin concentrations between the plasma and the beverage ingested. These profiles will be also helpful for clarifying various biological and pharmacological effects of green tea catechins [1–6], such as their effect on obesity. When the present method developed to analyze the free forms of catechins in the plasma is used with enzymatic treatments [12,21,22,31,32], it would also simply provide with the levels of the conjugated forms for the eight catechins.

In conclusion, a method has been established for analyzing eight green tea catechins (GC, EGC, C, EC, EGCG, GCG, ECG and CG) in human plasma after oral ingestion of a green tea beverage, which uses LC–ESIMS analysis following a preparative procedure for deproteinizing. The optimized LC–ESIMS provides a sensitive and specific determination of all the authentic eight catechins, and was used to analyze their levels in actual human plasma after the ingestion. The preparative procedure for deproteinizing enables us to simply analyze many plasma solutions that were prepared and subsequently placed within an autosampler overnight. Due to the lower overall recovery in this analysis, the measured quantitative values should be regarded as not absolute but relative ones. Using this method, we have detected and determined all eight catechins in plasma for the first time. It has been also suggested that extremely small amounts of the eight catechins orally ingested may be absorbed based on each absorptive property for the catechins. The method established in this study can be used for pharmacokinetic studies of green tea catechins after oral ingestion of a green tea

beverage, and should contribute to clarification of beneficial health effects, for example an anti-obesity effect, that prevent so-called lifestyle-related diseases.

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References

[1] C.S. Yang, Z.-Y. Wang, J. Natl. Cancer Inst. 85 (1993) 1038.
[2] T. Okuda, Y. Kimura, T. Yoshida, T. Hatano, H. Okuda, S. Arichi, Chem. Pharm. Bull. 31 (1983) 1625.
[3] K. Muramatsu, M. Fukuyo, Y. Hara, J. Nutr. Sci. Vitaminol. 32 (1986) 613.
[4] N. Matsumoto, F. Ishigaki, A. Ishigaki, H. Iwashima, Y. Hara, Biosci. Biotech. Biochem. 57 (1993) 525.
[5] T. Murase, A. Nagasawa, J. Suzuki, T. Hase, I. Tokimitsu, Int. J. Obes. 26 (2002) 1459.
[6] T. Nagao, Y. Komine, S. Soga, S. Meguro, T. Hase, Y. Tanaka, I. Tokimitsu, Am. J. Clin. Nutr. 81 (2005) 122.
[7] R. Seto, H. Nakamura, F. Nanjo, Y. Hara, Biosci. Biotech. Biochem. 61 (1997) 1434.
[8] Z.-Y. Chen, Q.Y. Zhu, D. Tsang, Y. Huang, J. Agric. Food Chem. 49 (2001) 477.
[9] I. Ikeda, M. Kobayashi, T. Hamada, K. Tsuda, H. Goto, K. Imaizumi, A. Nozawa, A. Sugimoto, T. Kakuda, J. Agric. Food Chem. 51 (2003) 7303.
[10] J.J. Dalluge, B.C. Nelson, J. Chromatogr. A 881 (2000) 411.
[11] H. Horie, K. Kohota, J. Chromatogr. A 881 (2000) 425.
[12] M.-J. Lee, Z.-Y. Wang, H. Li, L. Chen, Y. Sun, S. Gobbo, D.A. Balentine, C.S. Yang, Cancer Epidemiol. Biomarkers Prev. 4 (1995) 393.
[13] Y. Ho, Y.-L. Lee, K.-Y. Hsu, J. Chromatogr. B 665 (1995) 383.
[14] T. Unno, T. Takeo, Biosci. Biotech. Biochem. 59 (1995) 1558.
[15] T. Unno, K. Kondo, H. Itakura, T. Takeo, Biosci. Biotech. Biochem. 60 (1996) 2066.
[16] K. Nakagawa, T. Miyazawa, Anal. Biochem. 248 (1997) 41.
[17] G. Maiani, M. Serafini, M. Salucci, E. Azzini, A. Ferro-Luzzi, J. Chromatogr. B 692 (1997) 311.
[18] S. Carando, P.-L. Teissedre, J.-C. Cabanis, J. Chromatogr. B 707 (1998) 195.
[19] M. Harada, Y. Kan, H. Naoki, Y. Fukui, N. Kageyama, M. Nakai, W. Miki, Y. Kiso, Biosci. Biotech. Biochem. 63 (1999) 973.
[20] K. Umegaki, A. Sugisawa, K. Yamada, M. Higuchi, J. Nutr. Sci. Vitaminol. 47 (2001) 402.
[21] H.-H.S. Chow, Y. Cai, D.S. Alberts, I. Hakim, R. Dorr, F. Shahi, J.A. Crowell, C.S. Yang, Y. Hara, Cancer Epidemiol. Biomarkers Prev. 10 (2001) 53.
[22] A. Kotani, N. Miyashita, F. Kusu, J. Chromatogr. B 788 (2003) 269.
[23] J. Wu, W. Xie, J. Pawliszyn, Analyst 125 (2000) 2216.
[24] D.J. Zeeb, B.C. Nelson, K. Albert, J.J. Dalluge, Anal. Chem. 72 (2000) 5020.
[25] Q. Wu, M. Wang, J.E. Simon, Anal. Chem. 75 (2003) 2440.
[26] J.J. Dalluge, B.C. Nelson, J.B. Thomas, M.J. Welch, L.C. Sander, Rapid Commun. Mass Spectrom. 11 (1997) 1753.
[27] M. Takino, S. Daishima, K. Yamaguchi, T. Nakahara, Analyst 128 (2003) 46.
[28] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[29] G.K. Poon, J. Chromatogr. A 794 (1998) 63.
[30] K. Kudo, N. Ikeda, A. Kiyoshima, Y. Hino, N. Nishida, N. Inoue, J. Anal. Toxicol. 26 (2002) 119.
[31] M.-J. Lee, S. Prabhu, X. Meng, C. Li, C.S. Yang, Anal. Biochem. 279 (2000) 164.
[32] M.K. Piskula, J. Terao, J. Nutr. 128 (1998) 1172.